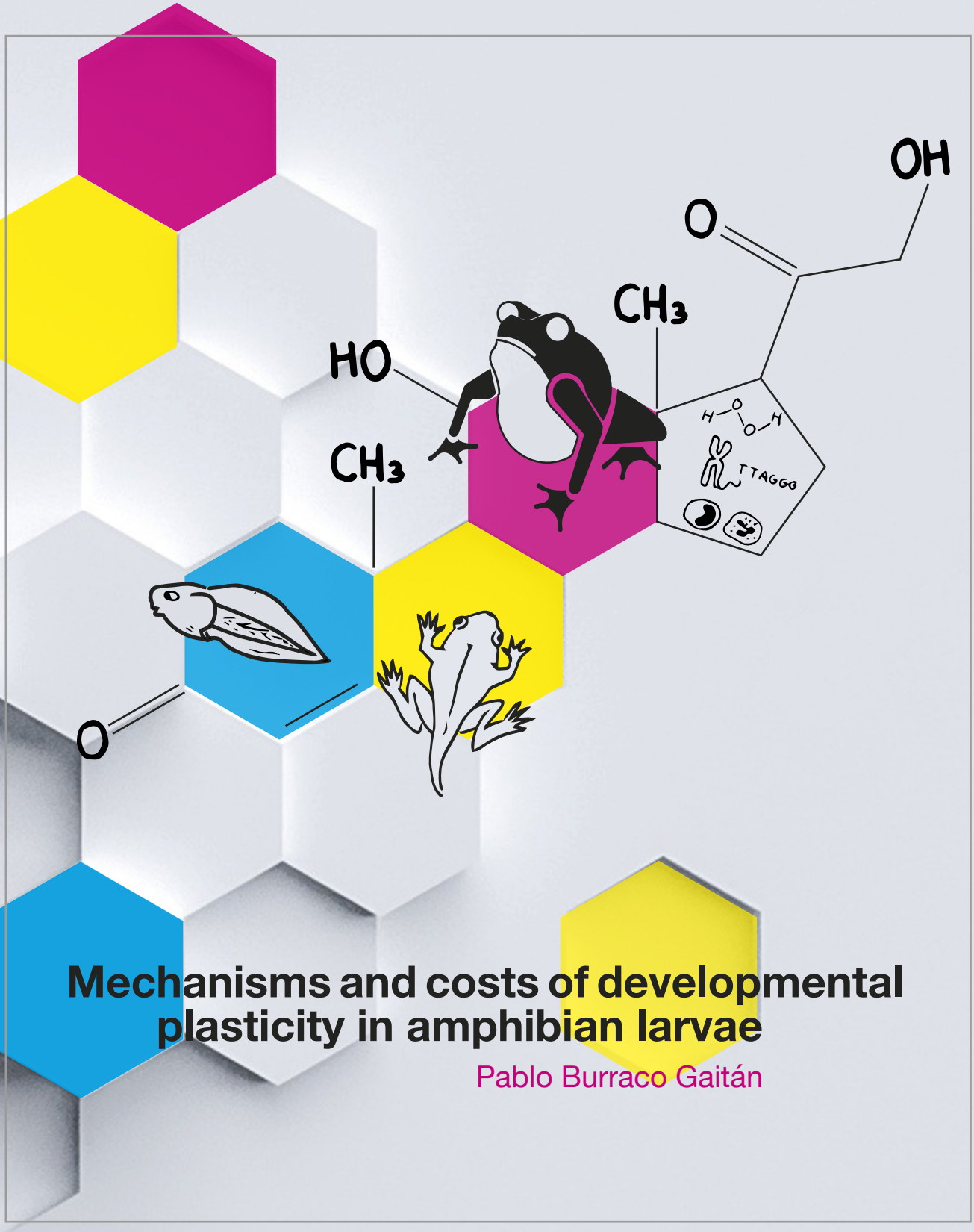

Mechanisms and costs of developmental plasticity in amphibian larvae

Pablo Burraco Gaitán

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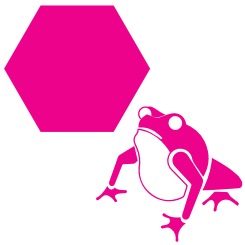

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Mechanisms and costs of developmental plasticity in amphibian larvae

Memoria presentada por el licenciado en Biología

Pablo Burraco Gaitán

para optar al título de Doctor por la
Universidad Pablo de Olavide de Sevilla.

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CERTIFICAN

Que los trabajos de investigación desarrollados en la Memoria de Tesis Doctoral: *Mechanisms and costs of developmental plasticity in amphibian larvae*, son aptos para ser presentados por el licenciado en Biología por la Univesidad de Sevilla, Pablo Burraco Gaitán ante el Tribunal que en su día se designe, para aspirar al Grado de Doctor por la Universidad Pablo de Olavide.

Y para que así conste, y en cumplimiento de las disposiciones legales vigentes, extendemos el presente certificado a 4 de mayo de 2017.

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Mechanisms and costs of developmental plasticity in amphibian larvae

“A todos los que guiaron a mi genética a transcribirse en modo de felicidad”

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Summary

Nature is complex and organisms commonly need to rapidly be able to detect and respond to environmental inputs in order to increase their survival odds. The ability of a given genotype to alter its morphology, behavior or development against changing environments is known as phenotypic plasticity, which is adaptive when the induced phenotypes confer increased fitness in the altered environment. Adaptive plasticity favors phenotypic diversity and increases population viability, as well as facilitates the maintenance of genetic variation reducing the severity of bottleneck events during rapid environmental change, and also by shielding genetic variants from selection. In particular, phenotypic plasticity is essential for species with low vagility and high philopatry, as is the case of most amphibian species.

The life-cycle of amphibians is often a complex one that includes an aquatic larval stage that gives rise to a terrestrial stage through metamorphosis. Metamorphosis is thus a key ontogenetic switch point that entails vast anatomical, physiological and ecological changes in the organism. The timing and body condition at which metamorphosis occurs largely determine the likelihood of survival in larval and juvenile amphibians. Growth and development are remarkably decoupled over long periods of the amphibian larval

ontogeny. This allows amphibian larvae to grow without advancing in development under benign conditions of high food availability, reduced competition, and abundant water, or else accelerate development at the expense of truncating growth when conditions worsen, as when at risk of pond drying. Such fine-tuning of growth and development relies on the ability of amphibian larvae to sense their environment and is regulated by neuroendocrine pathways, which can activate/repress multiple metabolic cascades, which in turn can involve short and long-term consequences for body condition and even life span. The main objective of this thesis is to understand the physiological mechanisms enabling developmental and growth plasticity in amphibians, and their consequences.

Firstly, several simultaneous experiments were conducted to study the physiological alterations inflicted by common potential external stressors on amphibian larvae. In particular, we studied the effect of varying levels of salinity, herbicide, water pH, types of predators, and temperature in spadefoot toad larvae (*Pelobates cultripes*) evaluating their changes in corticosterone level, metabolic rate, activity of various antioxidant enzymes, lipid peroxidation, and immune state. Most of the studied levels factors caused some physiological imbalances in tadpoles, although high levels of salinity and herbicide caused the most dramatic physiological alterations. Tadpoles showed decreased levels of corticosterone when exposed to native predators, congruent with the common reduction in foraging and metabolic activity in the presence of predators. Interestingly, however, tadpoles did not reduce corticosterone in the presence of invasive predators, indicating a lack of innate recognition. Furthermore, corticosterone and the antioxidant enzyme glutathione reductase were the most sensitive parameters against the studied factors, and hence good candidates for further use in physiological monitoring of natural populations.

Pond drying and predators are two of the main risks for amphibian larvae in temporary ponds. Physiological consequences of plastic responses in tadpoles after being exposed to both factors may allow us to understand short-term consequences of the developmental alterations induced, as well as to predict their long-term effects. We crossed the presence/absence of predators with permanent and drop-down water levels in a 2x2 experimental design simulating natural conditions in large outdoor mesocosms to test for induced changes in life-history traits, and also assessed their effects on fat reserves, oxidative stress, and telomere length of the surviving *Pelobates cultripes* juveniles that survived to both risks. Tadpoles accelerated their development in response to decreased water level, but at the expense of metamorphosing smaller and greatly depleting their fat reserves. Cellular oxidative stress due to developmental acceleration was successfully buffered by increased antioxidant enzyme activity, and telomere length remained unchanged. On the other hand, predators greatly reduced larval density, which relaxed competition and allowed survivors to develop fast, grow bigger and accumulate fat. However, tadpoles showed signs of oxidative stress and experienced telomere shortening. Telomere length is reduced during cell replication, and telomere shortening is known to be associated with senescence and reduced life span. Therefore, regarding body size and fat reserves at metamorphosis, developmental acceleration in response to pond drying compromises short-term survival, although its consequences are reversible in the long run. In turn, oxidative stress and telomere shortening due to rapid growth when tadpoles survived predators are likely reduce their long-term survival.

Even within species, organisms do not respond phenotypically to the same extent against environmental inputs. In fact, natural

populations often harbor substantial variation in their degree of phenotypic plasticity. Theoretical studies suggest that the evolution of plasticity is limited by costs of maintaining the machinery needed to detect and respond to environmental cues. However, only a few studies have empirically detected maintenance costs of plasticity. In the third chapter of this thesis, we tested for physiological costs of maintaining developmental, growth, and morphological plasticity in *Pelobates cultripes* larvae in response to both pond drying and predators. For this purpose, we first determined the degree of plasticity of a total of twenty families (sibships) from various populations in response to these two environmental factors. Simultaneously, we assessed among families variation in body mass, fat reserves, metabolic rate, antioxidant enzyme activities, lipid peroxidation, reduced/oxidized glutathione, and immune state under benign control conditions. We tested the existence of costs of plasticity by testing for an association between the degree of plasticity of each family and the physiological markers of stress. We found maintenance costs of plasticity associated to the degree of developmental and growth plasticity induced by predators, in terms of increased glutathione reductase activity and granulocyte to lymphocyte ratio, respectively. Morphological plasticity in response to both pond drying and predators were also linked to levels of antioxidant enzymes, lipid peroxidation, immune state or growth. Also, we detected trade-offs between the developmental responses of larvae to each factor so that genotypes (families) that readily accelerated development in response to pond drying were not effective in delaying metamorphosis in the presence of predators. Such a trade-off suggests possible constrains on the evolution of adaptive plasticity to conflicting environmental stimuli.

Environmental heterogeneity can affect the degree of adaptive plasticity, which may also imply short and long-term consequences. In amphibians, the ability to accelerate development commonly results adaptive, since larvae usually inhabit pools in which water availability is seasonal and heterogeneous. Adaptive developmental plasticity is expected to evolve despite its possible costs when environmental heterogeneity precludes a single phenotype to maximize fitness across all conditions. Swedish *Rana temporaria* island populations show marked differences among populations in developmental rate and their responsiveness to pond drying so that populations with more variable durations in pond hydroperiod tend to show higher levels of developmental plasticity. In the fourth chapter, we study the physiological mechanisms and consequences of divergent developmental plasticity among some of these *R. temporaria* populations. We exposed larvae from six populations from three different island habitat types differing in their pool drying regime to simulated desiccation to determine their developmental plasticity. Populations from islands with only ephemeral pools showed higher developmental plasticity than populations from islands with permanent or with a combination of both types of ponds. Individuals from islands with ephemeral ponds experience physiological alterations indicative of physiological costs of increased plasticity, such as altered catalase and glutathione reductase activities, and reduced telomere length. Elevated antioxidant activities indicate metabolic costs associated to increased developmental plasticity which may also compromise the health and lifespan of individuals and the viability of those populations, as shortened telomeres suggested.

During the course of this thesis, we have also evaluated the suitability of some methodological aspects.

Appendix i. We evaluated the performance of three commonly used procedures for corticosterone determination using *Xenopus laevis* tadpoles: radioimmunoassay (RIA) in whole-body homogenates, enzyme immunoassay (EIA) on whole-body, and EIA on plasma. Each procedure presented advantages and disadvantages regarding sensitivity, the use of radioactivity, sampling size, or handling time. RIA is preferred in small-bodied animals from which blood cannot be obtained. EIA in plasma resulted a good non-radioactive alternative when blood sampling is possible. EIA on whole-body homogenates was the less sensitive procedure, although it may be a non-radioactive useful alternative to assess qualitative changes in corticosterone in small individuals when considerable differences are expected.

Appendix ii. Immune response in amphibians has been commonly evaluated through indirect methods like phytohemagglutinin (PHA) injections or by direct like cell counts from blood smears. Here, we validated immunological evaluations in amphibians by means of flow cytometry. The immunological state of *Pelobates cultripes* tadpoles were experimentally altered by exposing them to exogenous corticosterone. Then, leukocyte proportions were quantified through both blood smears and flow cytometry. Both techniques showed similar patterns of leukocyte proportions. Once validated, flow cytometry also allowed quantification of changes in absolute number of leukocytes. The suitability of both techniques attending to accuracy, body size requirements, or the useful in field studies was also discussed.

The results obtained in this thesis highlight the key role of physiological mechanisms in amphibian larvae plasticity and in its evolution. Therefore, for a holistic knowledge of ecological and evolutionary process results essential to understand the physiology underlying them.

Resumen [in spanish]

La naturaleza es compleja y los organismos generalmente tienen la capacidad de detectar los cambios ambientales y de responder frente a ellos para así aumentar sus probabilidades de sobrevivir. La habilidad de un determinado individuo o genotipo para alterar su morfología, comportamiento o desarrollo frente a ambientes cambiantes es conocida como plasticidad fenotípica, la cual es adaptativa cuando aumenta la eficacia biológica de los individuos. La plasticidad adaptativa favorece la diversidad fenotípica y aumenta la viabilidad poblacional. Además facilita el mantenimiento de la variación genética, reduciendo la severidad de eventos de *cuello de botella* que se producen durante rápidos cambios ambientales, así cómo también blinda a las diferentes variantes genéticas frente a procesos de selección. En particular, la plasticidad fenotípica es esencial para especies con poca capacidad de dispersión y alta filopatría, como ocurre en la mayoría de especies de anfibios.

El ciclo de vida de los anfibios es a menudo complejo, incluyendo una fase larvaria acuática que a través de un proceso de metamorfosis da paso a una fase terrestre. La metamorfosis es un momento fundamental en la ontogenia de los anfibios y conlleva importantes cambios anatómico, fisiológicos y ecológicos en los organismos. La condición corporal de los individuos durante

metamorfosis y después de la misma determinan en gran medida sus probabilidades de sobrevivir. Sin embargo, crecimiento y desarrollo están notablemente desacoplados durante largos periodos de la ontogenia larvaria. De esta manera, las larvas pueden crecer sin avanzar en su desarrollo cuando se exponen a condiciones benignas tales como alta disponibilidad de comida o baja competencia intra o interespecífica, mientras que tienen la capacidad de acelerar el desarrollo a expensas de un menor crecimiento cuando las condiciones empeoran, como por ejemplo frente a un riesgo evidente de desecación de las charcas. Esta capacidad de ajustar crecimiento y desarrollo se basa en la habilidad de las larvas de percibir cambios ambientales. Las respuestas a estos cambios se regulan a través de rutas neuroendocrinas que activan o reprimen diversas cascadas metabólicas, lo que en última instancia puede conllevar serias consecuencias a corto y largo plazo tanto para la condición corporal de los individuos como para su esperanza de vida. El principal objetivo de esta tesis es entender los mecanismos fisiológicos involucrados en los cambios plásticos en el desarrollo en anfibios, así como sus consecuencias.

En primer lugar, se llevaron a cabo varios experimentos de manera simultánea en los que se estudiaron las alteraciones fisiológicas producidas en respuesta a factores potencialmente estresantes para larvas de anfibios. En concreto estudiamos el efecto de varios niveles de salinidad, herbicida, pH del agua, depredadores y temperatura en larvas de sapos de espuela (*Pelobates cultripes*), a partir de la evaluación de cambios en los niveles de corticosterona, tasa metabólica, actividad de varias enzimas antioxidantes, peroxidación lipídica y estado inmunológico. La mayoría de los niveles de los factores estudiados causaron desequilibrios fisiológicos en los renacuajos, aunque en concreto

altos niveles de salinidad y herbicida son los que causaron las alteraciones más dramáticas. Además, observamos niveles más bajos de corticosterona en renacuajos expuestos a depredadores nativos, lo que resulta congruente con el descenso en la tasa de actividad y en metabolismo detectado en estudios previos en larvas de anfibios expuestas a depredadores. Sin embargo, los renacuajos no redujeron los niveles de corticosterona en presencia de depredadores invasores, lo que indica una inexistencia de reconocimiento innato. Además, observamos que la hormona corticosterona y la enzima antioxidante glutatión reductasa fueron los parámetros más sensibles en respuesta a los factores estudiados, por lo que parecen ser buenos candidatos para ser usados en evaluaciones fisiológicas de poblaciones naturales.

La desecación de las charcas y los depredadores son los dos principales riesgos para las larvas de anfibios que viven en charcas temporales. Las alteraciones fisiológicas de las respuestas plásticas en renacuajos después de ser expuestos a ambos factores podría ayudar a entender las consecuencias a corto plazo de dichas respuestas, así como a predecir efectos a largo plazo. Para ello diseñamos un experimento en el que cruzamos la presencia/ausencia de depredador con la exposición a condiciones de agua constante/desecación en un diseño de 2x2. Este experimento se realizó en mesocosmos (tanques experimentales de 500-L) en los que se simularon condiciones naturales, para así poder evaluar cambios en los rasgos de historia de vida de las larvas de anfibios (crecimiento y desarrollo) así como los efectos sobre las reservas grasas, estrés oxidativo y la longitud de telómeros en aquellos individuos que sobrevivieron a los diferentes tratamientos. Los renacuajos aceleraron su desarrollo en respuesta a desecación, aunque a expensas de metamorfosear con un menor tamaño corporal

y con muy pocas reservas grasas. Sin embargo, la actividad de las enzimas antioxidantes consiguió neutralizar el estrés oxidativo celular y no se observaron variaciones en la longitud de las regiones teloméricas. Por otro lardo, la presencia de depredadores redujo en gran medida la supervivencia de las larvas, lo que disminuyó las relaciones de competencia por los recursos, permitiendo que los individuos se desarrollaran rápido, crecieran más y acumularan más grasas. Sin embargo, los renacuajos mostraron signos de estrés oxidativo a nivel celular y, además, experimentaron una reducción en la longitud de los telómeros. Los telómeros son secuencias terminales de los cromosomas que sufren reducciones durante eventos de replicación celular pero también durante procesos de estrés lo que induce una senescencia acelerada. Es por ello que un acortamiento de estas regiones se asocia a un descenso en la esperanza de vida de los organismos. Por consiguiente, teniendo en cuenta el tamaño corporal y las reservas grasas tras metamorfosis, la aceleración del desarrollo en respuesta a la desecación de la charca compromete la supervivencia a corto plazo de los juveniles de anfibios, aunque estas consecuencias podrían ser reversibles a largo plazo. En cambio, el estrés oxidativo y el descenso en la longitud de los telómeros en respuesta a un crecimiento rápido en aquellas larvas que sobrevivieron a depredadores parecen indicar que su esperanza de vida a largo plazo se verá reducida.

Incluso dentro de una misma especie los organismos no responden en la misma medida frente a las señales ambientales que reciben. De hecho, las poblaciones naturales a menudo albergan variaciones substanciales en su grado de plasticidad fenotípica. Estudios teóricos sugieren que la evolución de la plasticidad está limitada por los costes de mantener la maquinaria necesaria para detectar y responder a los cambios ambientales. Sin embargo,

solo unos pocos estudios han detectado de manera empírica dichos costes. En el tercer capítulo de esta tesis evaluamos los costes fisiológicos de mantener la plasticidad en el desarrollo, crecimiento y morfología en larvas de *Pelobates cultripes* en respuesta a la desecación de las charcas y a depredadores. Para ello, determinamos en primer lugar el grado de plasticidad en respuesta a estos dos factores para un total 20 familias biológicas procedentes de tres poblaciones espacialmente próximas entre sí. Así mismo, y de manera simultánea, determinamos los niveles de diferentes parámetros fisiológicos en individuos de esas mismas familias, expuestos a condiciones benignas, es decir, a nivel alto y constante de agua, y sin señales de depredador. En concreto se midieron variaciones en peso, reservas grasas, tasa metabólica, actividad de enzimas antioxidantes, peroxidación lipídica, glutatión oxidado y reducido, así como el estado inmunológico de los individuos de cada familia. Para evaluar la existencia de costes de la mantenimiento de las respuestas plásticas realizamos asociaciones entre el grado de plasticidad de cada familia frente a los dos factores (desecación y depredadores) y los marcadores fisiológicos de estrés que fueron medidos para cada una de esas familias. Observamos costes de mantener respuestas plásticas en desarrollo y crecimiento frente a depredadores ya que familias más plásticas presentaron niveles más altos de la enzima glutatión reductasa y del ratio granulocito:linfocito, respectivamente. Así mismo, familias con la capacidad de modificar en mayor medida su morfología (más plásticas) en respuesta tanto a desecación como a la presencia de depredadores también presentaron costes asociados, tal y como indicaron altos niveles de enzimas antioxidantes, peroxidación lipídica, ratio granulocito:linfocito o crecimiento. Además, observamos un compromiso entre la

capacidad de acelerar el desarrollo frente a la desecación de las charcas y la capacidad de retrasarlo en respuesta a depredadores, lo que sugiere que existen restricciones en la evolución de la plasticidad adaptativa en respuesta a estímulos ambientales que requieren respuestas fenotípicas opuestas.

La heterogeneidad ambiental puede determinar el grado que adquieren las respuestas plásticas adaptativas. Esta evolución de las respuestas plásticas puede también implicar consecuencias a corto y largo plazo para los organismos y poblaciones. En larvas de anfibios la habilidad para acelerar el desarrollo resulta adaptativa normalmente, debido a que las larvas generalmente viven en charcas en las que la disponibilidad de agua es estacional y heterogénea. Es de esperar que la plasticidad adaptativa en el desarrollo evolucione a pesar de sus posibles costes cuando la heterogeneidad ambiental impide que un único fenotipo maximice su eficacia biológica para todas las condiciones ambientales posibles. Poblaciones de *Rana temporaria* que habitan en un sistema de islas suecas muestran marcadas diferencias en su plasticidad en el desarrollo en respuesta a la desecación de las charcas, de manera que poblaciones expuestas a condiciones más variables de desecación tienden a tener una mayor plasticidad en el desarrollo. En el cuarto capítulo estudiamos los mecanismos y consecuencias a nivel fisiológico de la evolución divergente de la plasticidad en el desarrollo de estas poblaciones de *Rana temporaria*. Para ello expusimos a condiciones simuladas de desecación a individuos de seis poblaciones que provenían de tres hábitats diferentes, los cuales difieren en el régimen de desecación de sus charcas, y determinamos su capacidad para acelerar el desarrollo. Aquellas poblaciones pertenecientes a islas que sólo contaban con charcas efímeras mostraron una mayor plasticidad en

el desarrollo en comparación con poblaciones de islas que cuentan únicamente con charcas permanentes o con una combinación de ambos tipos de charcas. Individuos de islas que sólo poseen charcas efímeras presentaron alteraciones fisiológicas que podrían indicar costes asociados a tener la capacidad de responder de manera más plástica a procesos de desecación, como indicarían los cambios medidos en niveles de catalasa y glutatión reductasa, así como telómeros más cortos. Un aumento en la actividad de las enzimas antioxidantes indicarían costes metabólicos a nivel celular, lo que podría comprometer la salud y la esperanza de vida de los individuos, así como la variabilidad de las poblaciones, sugerido por una menor longitud de telómeros en estos individuos.

Durante el transcurso de esta tesis también hemos evaluado algunos aspectos metodológicos.

Apéndice i. Se evaluó la idoneidad de tres procedimientos comúnmente usados para determinar niveles de corticosterona usando renacuajos de *Xenopus laevis*: radioinmunoensayo (RIA) en homogenado de individuos, enzimoinmunoensayo (EIA) en homogenado de individuos y EIA usando plasma. Cada procedimiento presentó ventajas y desventajas con respecto a su sensibilidad, uso de radioactividad, tamaño muestral o tiempo de manejo de las muestras. RIA fue técnica más apropiada para ser usada en individuos pequeños en los cuales no es posible obtener suficiente muestra de sangre. EIA en plasma resultó ser una buena alternativa al uso de radioactividad, siempre y cuando sea posible obtener el volumen requerido de sangre. EIA en homogenado fue el procedimiento menos sensible a cambios en los niveles de la hormona, sin embargo podría ser una buena alternativa al uso de radiactividad en individuos de pequeño tamaño, aunque sólo cuando se esperen diferencias considerables en los niveles de la hormona.

Apéndice ii. La respuesta inmune en anfibios se ha evaluado frecuentemente a través de métodos indirectos tales como inyecciones de fitohemaglutinina (PHA), o bien a través de métodos de conteos directos a partir de frotis sanguíneos. Aquí hemos realizado una evaluación inmunológica a partir del uso de citometría de flujo. El estado inmunológico de larvas de *Pelobates cultripes* fue alterado experimentalmente a través de la exposición a corticosterona añadida de manera exógena al agua. A continuación se cuantificó la proporción de leucocitos a través de frotis sanguíneos y por citometría de flujo. Ambas técnicas mostraron patrones similares de las proporciones leucocitarias. Una vez validado el uso de citometría de flujo para medir la respuesta inmune en larvas de anfibios también cuantificamos cambios en el número absoluto de leucocitos. Así mismo discutimos la idoneidad de cada técnica con respecto a su precisión, volumen de muestra necesario o la posibilidad de ser usadas en estudios de campo.

Los resultados obtenidos en esta tesis enfatizan el papel clave de los mecanismos fisiológicos en la plasticidad de las larvas de anfibios, así como en la evolución de dichas respuestas plásticas. Por lo tanto, para un conocimiento holístico de procesos ecológicos y evolutivos resulta esencial entender la fisiología que los subyacen.

Introduction

Phenotypic plasticity in driving evolution

Individuals of a vast majority of animal and plant species have the property to modify some of their traits when detecting environmental changes, a property known as 'phenotypic plasticity'. The type and extent of plastic responses are specific of each species and environmental input (Pigliucci et al. 2006). Hence, a given environment can induce a plastic trait that not necessarily is expressed under other environment, as well a particular environmental change might induce more than a single plastic response. Plasticity is typically depicted using reaction norms, which are lines with a particular slope that indicate the extent of the phenotypic response expressed by a given genotype exposed to two or more environments. Some plastic responses are adaptive, i.e. when plasticity increases the individuals' fitness in comparison with fitness experienced by non-plastic individuals exposed to the same environmental condition. However, plasticity can be also non-adaptive or maladaptive when responses present unavoidable constraints imposed by molecular or developmental alterations (Sultan, 1995). The implications of non-adaptive responses are still under debate since it could inhibit adaptation but also might increase the strength of selection (Fitzpatrick, 2012; Ghalambor et al. 2015).

The evolutionary significance of phenotypic plasticity is being increasingly established. Plasticity facilitates the maintenance of genetic variation by, for example, shielding genetic variations from selection and moderating the effect of bottlenecks (Draghi and Whitlock 2012; Gomez-Mestre and Jovani, 2013). Phenotypic plasticity has also an important role in promoting divergence among populations, and also in the establishment of new species (Price et al. 2003; Pfennig et al. 2010; Davidson et al. 2011). For instance, it is well known the role of phenotypic plasticity in processes of sympatric speciation of cichlid fish species in Victoria Lake. In this lake, different disruptive selective forces favor a rapid speciation of sister fish species, which is initiated when subpopulations occupying the different microhabitats plastically alter key phenotypes such as body depth or eye length (Magalhaes et al. 2009).

Phenotypic plasticity increases phenotypic diversity and forms the basis for speciation in populations expressing moderate levels of plasticity (Pfennig et al. 2010). On the other hand, high levels of plasticity can impede selection by dampening the effects of selection for novel genetic variants (Price et al. 2003). Environmental heterogeneity plays a key role in determining the extent of adaptive phenotypic plasticity. Selection under heterogeneous environments favors the evolution of plasticity whereas homogenous conditions would tend to reduce plasticity (Gomez-Mestre and Jovani, 2013; Chevin and Lande, 2015). Also, associated costs might explain decreases in the extent of plasticity and to limit its evolution under inducing environments (Lind and Johansson, 2007). Changes in gene frequency on the regulation, form or consequences of a trait under selection, result in genetic accommodation, i.e. evolutionary change of phenotypic plasticity (West-Eberhard, 2003). Adaptive loss of plasticity under homogeneous conditions may eventually

result in the trait becoming under strict genetic control giving up its ancestral environmental sensitivity, in a particular case of genetic accommodation known as genetic assimilation. Genetic assimilation was first tested by Waddington (1942), in an experiment in which a *Drosophila melanogaster* population increased the frequency of a novel plastic phenotype (a second thorax) favored by the permanent exposure to homogeneous environmental conditions. After only 20 generations, flies expressed the novel form of the trait even in the absence of the environmental input. Waddington introduced the term 'canalization', defined as the ability of a genotype to produce the same phenotype regardless of its environmental variability. Hence, a phenotype is canalized when the threshold to activate the plastic response is no longer needed. Waddington's experiments denoted that plasticity is heritable and therefore genetically defined, which makes essential the understanding of the mechanisms underlying plasticity.

Costs and limits of plasticity

Plasticity is commonly different among genotypes of the same species even when such response is adaptive. The evolution of environmentally induced responses may be impeded by a series of evolutionary constraints or costs that limit and shape plasticity expression (DeWitt et al. 1998; Auld et al. 2010). Adaptive plasticity requires mechanisms to detect and respond to external stimuli which are subjected to natural selection (DeWitt et al. 1998; Agrawal et al. 2002). Maintenance and activation of those sensory and regulatory mechanisms is likely to be physiologically demanding, and if so, they could cause an impediment to the evolution or persistence of adaptive plasticity in natural populations. Plasticity could thus be partially

limited by facultative costs of maintaining sensory and regulatory machinery to respond, by costs of acquiring information from the environment (DeWitt, 1998, Auld et al. 2010), and/or by genetic costs such as pleiotropic, overdominance or epiptasis effects (Pigliucci and Schmitt 1999; Pigliucci 2005). If plasticity is fixed, costs are not expected to exist or are expected to be low, as for example under a scenario in which selective forces for a given plastic trait disappear, or after a canalization process that would reduce the threshold needed to detect and then to respond to changing environments. Some authors suggest that those maintaining costs of plasticity might be purged or reduced by selection (Dechaine et al. 2007; Van Burskirk and Steiner, 2009). Additionally, the costs of producing a plastic trait would also limit the evolution of such trait since plasticity necessarily involves a phenotypic restructuring at any level. Therefore, maintenance costs are inherent to plastic genotypes whereas production costs are only 'paid' when the plastic trait is induced, thus the ecological and evolutionary relevance of each type of cost is different (Callahan et al. 2008).

Maintenance costs of plasticity involve a fitness reduction in organisms even under benign conditions just for having the ability of being more plastic for a particular trait. However, although theoretical studies indicate the importance of costs in the evolution of plasticity, and a few empirical studies have successfully assessed plasticity costs, the relevance and magnitude of maintenance costs still remains unclear. Hence, it has been suggested that costs are mild probably because selection acts diminishing them. Also, some authors argue that costs are context-dependent and/or are high only under stressful environments (Van Burskirk and Steiner, 2009) or in species with large brains or with complex immune responses (Snell-Rood, 2012). However, costs could be frequently under or overestimated,

as it is difficult to experimentally replicate ecological contexts, which might produce non-realistic plastic responses (Agrawal 2001). Thus, plasticity costs determination might be influenced by the methods used to assess them, which might bias the conclusions regarding the role of costs in the evolution of plasticity (Kleunen and Fischer, 2005). Regarding this, the use of physiological, genomic, and epigenetic tools should help to determine accurately the existence of maintenance costs. In particular, Auld et al. (2010) indicated that physiological studies might provide adequate tools to evaluate the maintenance costs of plasticity. Actually, most of neurological and genetic alterations regulating plasticity ultimately involve physiological alterations such as metabolic shifts or changes in enzyme production or activity, as will be discussed below.

Production costs are those that organisms pay when a plastic trait is expressed. One classical example is the facultative expression of horns in males of at least seven beetle families. Frequently, only large males express fully developed horns whereas smaller males only develop rudimentary or even absent horns (Emlen et al. 2005; Kijimoto et al. 2013). In this example, if developing horns were costly, then males with fully developed horns would pay a physiological cost whereas males with absent or rudimentary horns would not. Of course, the extent to which the costs of producing horns are compensated or not by the associated fitness gain in terms of, for example, increased odds of mating or access to high quality females would determine the evolution of horn plasticity for that group of organisms.

Plasticity can be also limited by several factors beyond costs, which were summarized by DeWitt (1998) and later re-evaluated by Auld et al. (2010). Firstly, plasticity would be limited by the reliability of inputs that organisms perceive from the environment. Also, plasticity can vary seasonally and phenotype-environment matching sometimes

can only occur in a minimal lag-time. Likewise, is common that the ability to produce extreme phenotypes is restricted to a particular developmental range as well the induction of plastic responses during early developmental stages might limit responses later in development (Weinig and Delph, 2001).

Molecular approaches to phenotypic plasticity

Animal with central nervous system have the ability to rapidly sense and respond to environmental changes through gene expression changes and physiological alterations that lastly develop the phenotypic outcomes. However, the knowledge of mechanisms underlying phenotypic plasticity is still incomplete despite some plastic responses have been molecularly addressed in detail, as for example stomata movements, growth and development adjustments, or castes production in social insects (Schlichting and Smith, 2002). In addition, to fully address the machinery of plasticity results complicate since pathways involving plasticity include multiple down and up-regulations. For instance, Morris et al. (2013) described changes in gene expression in threespine stickleback populations (*Gasterosteus aculeatus*) exhibiting several plastic responses when exposed to their thermal tolerance extremes. The thermal tolerance experienced by populations of *G. aculeatus* allows organisms to inhabit both marine (ancestral populations) and freshwater (derived populations) habitats along the southern coast of British Columbia (Canada). Morris et al. (2013) estimated gene expression plasticity for 14,000 genes out of which 5,000 genes were similarly plastic in both populations. However they found that, overall, freshwater populations exhibited significantly more genes with plastic expression than marine populations. Studies like this provide molecular evidences

of the role of plasticity in colonization and adaptation to new environments. However, methodological and analytical limitations make still unfeasible to fully understand the regulatory network of such responses. On the other hand, the evaluation of epigenetic mechanisms is also increasingly being incorporated to phenotypic plasticity studies. Knowing the regulatory mechanisms of plasticity -including non-coding transcripts and transposable elements- will help to elucidate mechanisms controlling inducible traits.

For plasticity studies, it is also essential to consider physiological mechanisms in order to fully understand the limits, costs and constraints of plasticity evolution. Nowadays, there is an increasing use of physiology in evolutionary studies. Physiological mechanisms underlying some relevant plastic traits have been studied in depth in different taxa. For example, observed differences in the extent of developmental and growth plasticity in some species of insects are basically controlled by four hormones -insulin, juvenile hormone, prothoracicotropic hormone, and ecdysone- which at the same time regulate sexual size dimorphisms (Stillwell et al. 2010). Many of the studies on insects' plasticity have been conducted in the tobacco hornworm, *Manduca sexta*, describing the endocrine mechanisms of growth and developmental reaction norms against different environments (Davidowitz et al. 2004; Davidowitz and Nijhout, 2004). Those endocrine pathways are highly complex in insects and are sensitive to environmental conditions, as for example to nutritional conditions. Alterations in nutritional conditions can affect insulin secretion that regulates cell proliferation and protein synthesis, and lastly growth and body size of insects (Shingleton et al. 2005; Edgar, 2006). Also, insulin can interact with the juvenile hormone and with ecdysone, which can affect the expression of other traits (Stillwell et al. 2010). Similarly to the endocrine regulation of insect development and

growth, amphibian development is regulated by a series of hormones whose production is environmentally sensitive. Amphibians have the ability to detect slight environmental changes such as the increase of water temperature or the decrease of pond water level. Most of these changes increase the production of corticotropin-releasing hormone that stimulates the hypothalamo-pituitary-thyroid (HPT) and hypothalamo-pituitary-interrenal (HPI) axes. The stimulation of HPT and HPI axes results in increases of whole-body thyroid hormone, thyroxine, triiodothyronine, and glucocorticoids such as corticosterone (Denver, 1997; Boorse and Denver, 2003). An elevated content of these hormones and particularly of thyroid hormone and corticosterone accelerates larval development and morphogenesis, allowing them to complete metamorphosis earlier (Denver, 2009; Gomez-Mestre et al. 2013). Moreover, differences in the levels of these hormones and in metabolic rate explain differences in developmental plasticity observed in closely-related species of spadefoot toads, suggesting genetic accommodation of the regulatory mechanisms of developmental rate in response to decreased water level (Kulkarni et al. 2017).

In some cases, the same physiological pathway controls several plastic responses. For instance, the juvenile hormone has a key role in insects by modulating several plastic traits such as caste determination in eusocial species (Watanabe et al. 2014; Korb, 2015), wing polymorphisms in aphids (Schwartzberg et al. 2008), or mandible size in beetles (Okada et al. 2012). Regarding the role of the juvenile hormone in the regulation of growth and nutrition in insects, some of these plastic responses might be nutrition-dependent characters (Gotoh et al. 2014). In some cases, plastic responses are regulated by the same mechanism but acting opposite directions. In this line, amphibian larvae increase their corticosterone levels when larvae

detect that ponds begin to dry-up, thus accelerating metamorphosis (Gomez-Mestre et al. 2013). In contrast, larvae seem to reduce their corticosterone levels when facing predators, probably to diminish their movements in order to avoid being detected by predators (Hossie et al. 2010; Burraco et al. 2016), although this hypothesis is still under debate (Maher et al. 2013; Joshi et al. 2016). Therefore, the activation or suppression of some endocrine pathways can regulate different and even opposite plastic responses, which might limit the evolution of other traits.

Other physiological mechanisms have been recently included in plasticity studies. A prolonged secretion of stress hormones commonly implies a state of chronic stress that might alter cellular metabolism and lastly damage biomolecules such as lipids, proteins or DNA (Therond, 2006). Specifically, enhanced cellular metabolism involves increased metabolite transport and elevated catabolism, which augment the production of reactive oxygen species (ROS). An excess of ROS is toxic for the cells. However, increased activity of specific antioxidant enzymes can buffer the impact of ROS by detoxifying them, hence stabilizing cellular metabolic functions. Similarly to antioxidant enzyme activity, cells can elevate the production of free radicals scavenger molecules, like reduced glutathione, in order to avoid oxidative damages. These molecules act as a first line of defense against reactive species (Masella et al. 2005). Cellular oxidative damage occurs when the activity of antioxidant enzymes together with scavenger molecules are not enough to buffer ROS production. Metabolic costs of plasticity could alter cellular homeostasis caused by an overproduction of ROS (Constantini et al. 2010). Oxidative stress can compromise health and reduce lifespan of individuals because responses to ROS impose important costs in terms of immunosuppression and high susceptibility to diseases (Costantini, 2014; Sebastiano et al. 2016).

On the other hand, the effect of oxidative stress may also affect the length of telomeres, the terminal regions of eukaryotes chromosomes. Telomeric regions experience shortenings with age, known as the 'end replication problem'. They can also be shortened under stress episodes because these regions are sensitive to DNA-damaging agents, which can cause end repair problems (Allsopp et al. 1995; Epel et al. 2004). Telomere shortening is a reliable indicator of individuals ageing (Barret et al. 2013; Shalev et al. 2013), although this is still under debate (Simons, 2015), hence its inclusion in ecological and evolutionary studies should allow a better understanding of fitness costs and long-term consequences of plasticity.

Objectives

The main objective of the present PhD thesis is to understand the physiological mechanisms and consequences of phenotypic plasticity in amphibian larvae. Phenotypic plasticity is essential for species with complex life cycles, as it is the case of most amphibian species. The life cycle of amphibians often includes an aquatic larval stage that gives rise to a terrestrial juvenile through metamorphosis. Larvae normally face multiple environmental changes and respond by plastically modifying their development and growth. This makes amphibians an ideal group to study mechanisms and consequences of developmental plasticity. In particular, for larval amphibians living in aquatic habitats, desiccation and predation are two of the major risks they have to face. Thus, in this thesis we analyze the plastic responses of amphibian larvae to these two important risks, the mechanisms enabling their plastic responses, and the physiological consequences of their developmental responses.

Specifically, the objectives of this thesis are:

1. To study possible physiological unbalances in terms of endocrine, metabolic, antioxidant, and immune alterations experienced by amphibian larvae exposed to non-lethal levels of many common aquatic stressors.

2. To evaluate short and long-term consequences of developmental and growth plasticity in amphibian larvae exposed to desiccation and predation risks, and to understand the effects of growth and developmental alterations on oxidative stress and telomere shortening, both associated to health and lifespan.

3. To test for physiological maintenance costs of developmental plasticity in amphibian larvae exposed to either habitat desiccation or predators, and also to study possible trade-offs between the plastic responses to each factor.

4. To determine the physiological changes underlying adaptive divergent degrees of developmental plasticity among island frog populations evolving under different pond desiccation regimes.

CHAPTER 1

Physiological stress responses in amphibian larvae to multiple stressors reveal marked anthropogenic effects even below lethal levels

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Abstract

Natural and anthropogenic disturbances cause profound alterations in organisms, inducing physiological adjustments to avoid, reduce or remedy the impact of disturbances. In vertebrates the stress response is regulated via neuroendocrine pathways, including the hypothalamic-pituitary-adrenal axis that regulates the secretion of glucocorticoids. Glucocorticoids have cascading effects on multiple physiological pathways, affecting metabolic rate, reactive oxygen species production, or immune system. Determining the extent to which natural and anthropogenic environmental factors induce stress responses in vertebrates is of great importance in ecology and conservation biology. Here we study the physiological stress response in spadefoot toad tadpoles (*Pelobates cultripes*) against three levels of a series of natural and anthropogenic stressors common to many aquatic systems: salinity (0, 6, and 9 ppt), herbicide (0, 1, and 2 mg/L acid equivalent of glyphosate), water acidity (4.5, 7.0, and 9.5), predators (absent, native, and invasive), and temperature (21, 25, and 29 ºC). The physiological stress response was assessed examining corticosterone levels, standard metabolic rate, activity of antioxidant enzymes, oxidative cellular damage in lipids, and immunological status. We found that common stressors substantially altered the physiological state of tadpoles. In particular, salinity and herbicide cause

dramatic physiological changes in tadpoles. Moreover, tadpoles reduced corticosterone levels in the presence of natural predators but did not do so against invasive predators, indicating lack of innate recognition. Corticosterone and the antioxidant enzyme glutathione reductase were the most sensitive parameters to stress in this study. Anthropogenic perturbations of aquatic systems pose serious threats to larval amphibians even at non-lethal concentrations, judging from the marked physiological stress responses generated and reveal the importance of incorporating physiological information onto conservation, ecological, and evolutionary studies.

Keywords: Amphibians; Corticosterone; Immune system; Metabolic rate; Oxidative Stress; Stress physiology

Introduction

Environmental disturbances, whether natural or anthropogenic, cause physiological alterations of individual organisms that allow them to reduce or avoid the impact of the stressors (Romero, 2004; McCue, 2010). Such physiological responses, however, may come at a cost and result in fitness trade-offs associated with reduced immune competence, delayed growth and maturity, and shorter lifespan (Bonier et al. 2009; Shalev et al. 2013). In some cases, such disturbances impose entirely novel challenges to which organisms need to adapt, as is the case with many pollutants or the introduction of invasive predators. Moreover, humans are also causing faster and more acute modifications of factors to which organisms may be naturally exposed within a narrower range, as in water acidification, salinisation, or global warming (Kaushal et al. 2005; Lafferty, 2009). Stressors can profoundly alter the physiology of organisms well before reaching lethal levels, conditioning key aspects of their behaviour, growth or reproductive performance.

In vertebrates, the stress response is regulated by a set of neuroendocrine pathways of which the hypothalamic-pituitary-interrenal (HPI) axis is the most studied. The HPI-axis modulates a hormonal cascade resulting in the activation of the interrenal gland and glucocorticoid (GC) production: corticosterone (CORT) in amphibians,

reptiles, and birds, and cortisol in most mammals and fish (Romero, 2004). GCs elicit the mobilization of energetic metabolic substrates (e.g. lipids; Peckett et al. 2011), which affect essential functions of the organism like reproduction, behaviour, and growth (Denver et al. 2002; Schoech et al. 2009; Kindermann et al. 2013), thus conditioning the transition between life-history stages (Crespi et al. 2013). Prolonged secretion of CORT has been associated with mobilization of energetic substrates and increased metabolic demands in multiple tissues (Peckett et al. 2011; Lattin and Romero, 2015), while resulting in reduced long-term survival (Bonier et al. 2009). GCs have cascading effects on multiple physiological pathways. Elevated GCs cause increased metabolic rate, involving overproduction of reactive oxygen species (ROS; Peckett et al. 2011) that often result in cellular damage (Circu and Aw, 2010). Such cellular damage, however, can be buffered by increasing the activity of antioxidant enzymes (Costantini et al. 2011; Gomez-Mestre et al. 2013). Additionally, GC are inmmunomodulators that can exert both negative and positive effects on the immune status of individuals (Franchimont, 2004) depending on the duration and intensity of the exposure to stress (Rich and Romero, 2005), although chronic exposure to high GC levels seem associated with immune deregulation (Padgett and Glaser, 2003).

Many amphibian species have complex life cycles with aquatic larvae, which are often exposed to a suite of biotic and abiotic natural stressors. Amphibians are also deeply impacted by human disturbances and indeed constitute the most threatened group of vertebrates (Hoffman et al. 2010). CORT regulation is a common stress response in anuran tadpoles to pond drying, pollutants, predators, acidification, or UV-B radiation (Glennemeier and Denver, 2001; Chambers and Belden 2009; Maher et al. 2013; Chambers et al. 2013; Burraco et al. 2013). However CORT regulation has many potential cascading

effects on other aspects of amphibian biology, and these are seldom studied. Here we analyze the physiological stress response in spadefoot toad tadpoles (*Pelobates cultripes*) against a series of natural and anthropogenic stressors common to many aquatic systems. We measure physiological parameters relevant for evaluating the stress response: CORT levels, standard metabolic rate (SMR), antioxidant enzymes activity, oxidative cellular damage, and immune status. We tested the physiological response against three levels of salinity, herbicide (glyphosate), pH, temperature, and also against natural and invasive predators. All factors included in this study are considered potentially stressful for tadpoles and some of them can be magnified by human activities at either global or local scales. High salinity results in reduced tadpole survival and delayed metamorphosis (Hopkins and Brodie, 2015). Herbicide exposure reduces amphibian diversity and alters the outcome of competition interactions (Relyea and Mills, 2001). Water acidity also reduces survival and slows down development in embryos and larvae, particularly at pH 4.5 or lower (Merilä et al. 2004). The introduction of novel predators may have a deep impact on local populations (Siesa et al. 2011), in part because native prey are very likely to fail to recognize novel predators and hence fail to produce antipredator defences, whether behavioural or morphological. Lastly, increased water temperature causes developmental acceleration, causing larvae to metamorphose smaller and with reduced hind limbs (Gomez-Mestre et al. 2010; Duarte et al. 2012).

We expected most experimental factors to affect CORT levels, since the HPI-axis is known to play a central role in amphibian stress responses (Denver, 2013). We also expected changes in metabolic rate to be affected by the experimental factors chosen. Moreover, hormonal and metabolic changes may alter the production of ROS,

which may cause oxidative damage unless dealt with, and therefore we also expected the activity of antioxidant enzymes to increase when metabolism itself was elevated. Also, increased GC secretion is tightly associated with the immune system as it results in increased neutrophils:lymphocytes ratio (Davis et al. 2008). Likewise, enhanced immune responses under stress have been shown to incur in increased metabolic costs (Råberg et al. 2002).

This study will allow us to compare the magnitude of the stress responses across multiple factors and assess the association among physiological alterations. Comprehensive physiological studies are needed comparing the intensity and amplitude of physiological responses both to novel environmental challenges and to the intensification of natural stressors to which organisms may already be adapted. Assessment of various aspects of physiological stress responses to multiple factors will identify interdependence among such responses and possibly unveil mechanisms underlying life-history trade-offs. Physiological analyses of responses to multiple factors are therefore key both to conservation and eco-evolutionary studies.

Material and methods

Animal collection and experimental setup

We collected spadefoot toad tadpoles (*Pelobates cultripes*) from three temporary ponds (80 from each location) within the Biological Reserve of Doñana National Park (March, 2011) and from two temporary ponds within the Sierra Norte Natural Park (April, 2012) both in southwestern Spain, to run five experiments (see below). All tadpoles were collected between 34-35 Gosner stages (Gosner 1960) and their weight was 2.50 ±.0.3 (s.e.) g. We also collected water beetle

larvae (*Dytiscus circumflexus*) and red swamp crayfish (*Procambarus clarkii*) in several ponds within the Biological Reserve. Both species are relevant tadpole predators but *D. circumflexus* larvae are native predators whereas the red swamp crayfish was introduced in the 1970's and have become a common invasive predator since (Diaz-Paniagua et al. 2014). Nevertheless, we still consider *P. clarkii* a novel predator since amphibians in the Park show lack of innate recognition of *P. clarkii*, as indicated by the inability of both *Pelophylax perezi* and *P. cultripes* to induce behavioural or morphological defences against crayfish, whereas they readily deploy such defences against native predators (Gomez-Mestre and Díaz-Paniagua, 2011). We found neither crayfish nor water beetle larvae at the ponds where the tadpoles were collected. This suggests (but does not grant) that the larvae included in the study were naïve to either kind of predator. Previous exposure to native predators could have partially induced phenotypic responses whereas exposure to invasive predators could have given tadpoles the chance to learn to recognize their cues, if paired with alarm cues from attacked conspecific tadpoles (Polo-Cavia and Gomez-Mestre, 2014). Tadpoles collected from natural ponds thus represent a conservative test regarding naïveté towards invasive predators. All tadpoles were acclimated for one week in 4L buckets (4 ind/bucket) filled with dechlorinated tap water in climate chambers set at 21 ºC and 12:12 light:dark cycle according to natural conditions in the field. Tadpoles were fed *ad libitum* with rabbit chow. Predators were maintained individually in 4L buckets.

We conducted five independent experiments, each one testing for physiological responses to exposure to different levels of each of five factors separately: salinity, herbicide, pH, predators, and temperature. Experiments were conducted in two consecutive breeding seasons. We pooled tadpoles from all clutches collected within each

season. Tadpoles collected from the Biological Reserve of Doñana in 2011 were used for salinity, pH, and predator exposure experiments, whereas those from the Sierra Norte Natural Park in 2012 were used for herbicide and temperature experiments. Groups of four tadpoles were kept in 3L buckets filled with carbon-filtered dechlorinated tap water. Experimental units were randomized within each experiment across shelves in a walk-in chamber set at constant 21 ºC and a 12:12 light-dark cycle. Water was renewed twice a week and tadpoles were fed *ad libitum* with rabbit chow. Each experiment had its own set of control replicates, which all had the same conditions: 21 ºC, pH 7, and herbicide-free freshwater without predator cues. We randomly assigned containers to experimental treatments. Treatments lasted for 10 days and were initiated after a one-week acclimation period in the climatic chambers under control conditions.

Stress factors

We selected three non-lethal levels for each stress factor: salinity (0, 6, and 9 ppt NaCl), herbicide (0, 1, and 2 mg/L of glyphosate), pH (4.5, 7.0, and 9.5), predators (absent, native, and invasive), and temperature (21, 25, and 29 ºC). The levels applied for the different factors were chosen based on previous knowledge of ranges commonly experienced by amphibians either in natural systems or in areas affected by human activities (Alvarez and Guerrero, 2000; Gomez-Mestre et al. 2003; Solomon and Thompson, 2003; Serrano et al. 2006, Diaz-Paniagua et al. 2014). We replicated 10 times each treatment, for a total of 150 experimental units and 600 tadpoles. Almost all individuals survived throughout the experimental procedure (94.67 %), confirming the non-lethality of the treatments chosen during the 10 days of exposure. Tadpoles allocated to the highest levels

of salinity, temperature, and herbicide were previously acclimated for three days at the intermediate levels, so they were exposed to the highest level for only seven days. Even short acclimation periods seem to be critical to allow enough time to mount an effective physiological response to acute stressors (Wu et al. 2014).

After 10 days, we randomly collected one tadpole per container and we measured their standard metabolic rate as described below. We also extracted blood for leukocyte determination from another tadpole randomly chosen from each container. Then, we collected the remaining tadpoles, we euthanized them individually by immersion in a lethal concentration of anesthetic (MS-222), and we randomly allocated one tadpole per experimental unit to CORT assay and oxidative stress assays.

Salinity experiment

To obtain the target salinity levels (0, 6, and 9 ppt) we added commercial sea salt (Instant Ocean – Aquarium system) as required for each treatment. To prevent possible osmotic shocks derived from direct transfer to 9 ppt (Wu et al. 2012) we acclimated tadpoles assigned to the 9 ppt treatment in a solution at 6 ppt for 3 days prior to onset of the experiment. We monitored salinity twice a week using an osmometer (WTW, model Multi 340i) and a refractometer (LABOLAN, model RHS-10). Salinity varied ± 0.2 ppt for 6 ppt and 9 ppt treatments, and did not vary for 0 ppt.

Herbicide experiment

We tested tadpoles' response to glyphosate, which is one of the herbicides most widely used in crop fields worldwide (Solomon and

Thompson, 2003). We used a stock solution containing 360 g/L of isopropylamine salt of glyphosate (Fortin, Industrial química key, S.A.). We used two different glyphosate concentrations: 1 and 2 mg/L acid equivalent. Glyphosate was made fresh before each water change to avoid possible glyphosate degradation since its half-life in water is 7-14 days (Giesy et al. 2000).

pH experiment

We obtained the target pH levels (4.5, 7.0, and 9.5) by adding either sodium carbonate (PQS) or sodium bisulphate (PQS) from concentrated stock solutions. We checked water pH daily with a pH meter (WTW, model Multi 340i), adjusting it as necessary. pH values varied by ± 0.4 regardless of the pH level.

Predator experiment

Predators were introduced in cages made of plastic cups (250 mL) with a mesh screen bottom that allowed water flow and cue diffusion. Buckets in the predator absent treatment contained empty cages. We surveyed the experiment daily and replaced any dead predators. We feed predators in external housing tanks to avoid confounding the detection of predator kairomones with detection of alarm cues from injured tadpoles in the experimental buckets.

Temperature experiment

We used individual aquarium heaters (25W) in each bucket to regulate temperature. Experimental units in the 21 ºC treatment also contained heaters but were switched off. We increased temperature

in a two-step process so that tadpoles assigned to the 29 ºC treatment were first maintained at 25°C for 3 days to allow tadpoles to acclimate. We verified the water temperature daily with a thermometer (RTD, Delta Ohm) and found it to be very stable. The temperature was constant and only varied ± 0.3 ºC in each level (21 °C, 25 ºC and 29 ºC).

Corticosterone assay

CORT levels were determined from whole-body homogenates by performing enzyme immunoassay (EIA; Burraco et al. 2015) using commercial kits (Cayman Chemical Company, USA). This procedure is a conservative test of CORT differences across treatments, as it has lower sensitivity than radioimmunoassay (RIA) or EIA on plasma samples (Burraco et al. 2015). However, it does not require use of radioactive isotopes and allowed us to keep the number of animals used in the study at a minimum given the high combination of factors and levels required (Burraco et al. 2015).

Tadpole homogenates were centrifuged at 4000 rpm at 4 ºC for 15 min. We took 50 µL from the resulting supernatant for EIAs. EIAs are quantitative assays based on competitive binding between the target hormone and a conjugated CORT tracer (CORT-acetylcholinesterase) for a limited number of CORT-specific sheep antiserum binding sites which bind to the rabbit polyclonal anti-sheep IgG previously attached to the well. Quantitative estimates were obtained by reading absorbance at 412 nm, and CORT concentrations were determined based on standard curves run in duplicate on each plate. Each sample was run by duplicate. The detection limit (80 % B/B0) for this kit is 30 pg/mL as indicated by the manufacturer and cross reactivity with other steroids is below 1 %.

Standard metabolic rate

We used an aquatic respirometer to measure standard metabolic rate (SMR) consisting in a set of ten flow-through cells (plexiglass cylinders, 44 m in diameter x 163 mm long cylinders) with twenty optical sensors. Two sensors flanked each chamber to simultaneously measure the oxygen concentration (mg/L) coming in and out of the chamber. We connected the optical sensors to an oxymeter (Oxy 10-PreSens, Germany) and we programmed it to record oxygen partial pressure every 15 seconds. The optical sensors used (optodes) do not consume oxygen during measurements, have long-term stability and their signal does not depend on the flow rate of the sample. We calibrated the respirometer at the same temperature as the experimental units experienced: 25 ºC and 29 ºC for the experimental temperature treatments, and at 21 ºC for all the rest. Calibration took place at least once daily using a sodium sulphite solution and oxygen saturated water to reach 0 and 100 % concentrations. From each bucket we introduced one random tadpole individually in each plexiglass chamber. All tadpoles were at Gosner stage 35 (Gosner, 1960). We recorded oxygen consumption for 25 min but discarded the first five minutes of the data series, considered as acclimation period of the animals to the chambers and SMR values were calculated as in Álvarez et al. (2006). Based on our previous experience, after 5 min tadpoles seems to behave normally and respirometer output is quite stable. In our experience 20 min of effective recording time provide reliable measurements compared to longer recordings on the same species. This procedure allowed us to compact the overall number of days required to collect all the data, hence avoiding the potential confounding factor of having later assayed tadpoles being more advanced in development than earlier assayed tadpoles. All trials were

conducted between 09.00 and 14.00 h to avoid circadian effects. Upon release from the chambers, tadpoles were blotted dry and weighed to the nearest 0.1 mg on a high precision balance (CP324S, Sartorius, Germany).

Oxidative stress

We quantified the activity of four antioxidant enzymes: catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR). We also quantified the cellular damage by measuring thiobarbituric acid substances (TBARS) formed during lipid peroxidation.

Upon completion of the experiment, we euthanized tadpoles in benzocaine 0.01 %, snap froze them in liquid nitrogen, and stored them at -80 ºC until the assays were conducted. We thawed the samples and dissected the specimens to remove the gut in order to avoid possible interferences with the assays. Then, samples were individually homogenized in a buffered solution (100 mM Tris-HCL with 0.1 mM mM EDTA, 0.1 % triton X-100, pH 7.8 and 0.1 mM PMSF, for the inhibition of proteolysis) using a homogenizer at 35,000 rpm (Miccra D-1). We mixed 1 g of tissue in 4 ml of homogenization buffer (1:4, w:v). The homogenated tissues were centrifuged at 14,000 rpm for 30 minutes at 4 ºC. We aliquoted the resulting supernatant into several 0.6 mL tubes and we cryopreserved it at –80 ºC. We determined the total protein content assessed to calculate the antioxidant enzymes activity by standard Bradford's method (Bradford, 1976).

We quantified CAT activity in terms of catalytic activity with an indirect method, according to Cohen and Somerson (1969). We used potassium permanganate ($KMnO_4$) that is an oxidizing agent and coloured compound which acts on the H_2O_2 (reducing agent) produ-

cing H_2O_2 and O_2. $KMnO_4$ is reduced producing a red product (absorbance read at a wavelength at 480 nm). We performed standard curves of commercial catalase (SIGMA-60634) and we determined absorbance at a wavelength of 480 nm five minutes after adding $KMnO_4$. We expressed the catalase activity as U/mg of total proteins. According to Cord and Fridovich (1969) we obtained the SOD activity levels by measuring the cythocrome C inhibition rate, produced by SOD: superoxide free radicals (O_2-) reduce the ferrocythocrome C (xantine-xantine oxidase enzymatic system) but in SOD presence this reaction is inhibited because of superoxide radicals produce hydrogen peroxide and molecular oxygen. One unit of SOD is defined as the amount of enzyme that inhibits the rate of reduction of ferrocytochrome C by 50 % at 25°C at 550 nm (Cord and Fridovich, 1969). We determined GPx activity as described Paglia and Valentine (1967). GPx converts hydrogen peroxide into water, but requires reduced glutathione (GSH) that is produced by GR through oxidized glutathione (GSSG) reduction. To quantified the GPx activity we measured NADPH oxidation by reading absorbance at a wavelength of 340 nm. We quantified GR activity following Cribb et al. (1989). We measured the change in absorbance at 340 nm due to NADPH oxidation, as described in the GPx assay. The formation of TBARS is due to lipid peroxidation and is increased during cellular damage processes. One product of lipid peroxidation is malondialdehyde (MDA), which reacts with thiobarbituric acid producing a red product absorbing at 535 nm. We measured TBARS concentration according to Buege and Aust (1978). We measured the optical density values for the blank and for the calibration curve. Then, we calculated the TBARS concentration (in nmol MDA/ml) from the absorbance of each sample, subtracting the blank values and comparing with the calibration values.

Immune status

We counted leukocytes to assess stress condition through direct cell observation from blood smears. Although a single observer recorded leukocyte proportion we estimated the variation between observers (CV=8.71 %). The CV intrasample was 6.99 %. The Blood was obtained via cardiac venipuncture with a 29G syringe (BD Micro-Fine Insuline U-100 0.5 ml) in tadpoles anesthetized with MS-222. The resulting blood smears were stained using Pappenheim method (May-Grünwald - Giemsa staining) and were fixed onto the glass slides with DPX (Eukitt Mounting Medium). We identified and counted the proportion of lymphocytes and granulocytes (basophils, neutrophils, and eosinophils) out of 100 white cells in each sample using a 10X ocular (total magnification: 1000X), using Oil DC, and a ZEISS Immersion microscope, model Zi.

Statistical analyses

We conducted all statistical analyses in R (version 2.14-1, R Development Core Team 2007). We tested for normality by means of Kolgomorov-Smirnov tests (lillie.test, package nortest version 1.0-3), and for homogeneity of variances with Barlett's tests (bartlett.test) and also through visual inspection of residuals. Otherwise, we used Akaike Information Criterion (AIC) to assess the goodness of fit of each model and chose the appropriate error distribution. When parametric assumptions were met, we used linear models with a Gaussian distribution and an identity link function. We used Gamma distributions where appropriate in generalized linear models with the glm function included in the MASS package (version 7.3-40). For SMR analysis we used body weight as a covariate to control for the effect

of body mass in oxygen consumption. CORT, GR, and GPx were also significantly affected by body mass although its explanatory power was very low ($R^2 < 0.05$) except for GR ($R^2 = 0.156$; $P < 0.001$). Therefore, we only included body mass as a covariate in SMR and GR analyses. CORT and SMR data were log-transformed to meet parametric assumptions. We tested for differences in the proportion of leukocytes by fitting generalized linear models with a binomial distribution. We conducted *post-hoc* tests (Tukey tests) using TukeyHSD function (multcomp package, version 1.2-13) when overall tests were significant to test for differences among treatments.

Results

Pelobates cultripes tadpoles exposed to non-lethal levels of salinity and glyphosate experienced changes in most of the physiological parameters measured, modifying CORT levels, metabolic rate, antioxidant enzymatic activity, and leukocyte counts (see Table 1). Furthermore, changes in pH, temperature, and predators also produced hormonal, enzymatic or immune alterations in spadefoot toad tadpoles. Because experiments were run in two consecutive breeding seasons including animals from two different locations, the absolute values were not directly comparable across all experiments. Consequently, and since each experiment had its independent control treatment at 21 °C and neutral water, we plotted the results for each experimental treatment as the relative change of each variable with respect to the control.

Responses to salinity

Increased salinity significantly increased CORT levels ($F_{2,22}$ = 3.856, P = 0.040; Fig. 1), with tadpoles in 9 ppt showing a 2.78-fold increased on average compared to tadpoles in 0 ppt and 6 ppt. High salinity also increased SMR ($F_{2,28}$ = 3.86, P = 0.035; Fig. 2). Tadpoles in 9 ppt increased on average their SMR by 2.41-fold, (P = 0.026) compared to the ones in 0 ppt. Salinity altered the activity of antioxidant enzymes, particularly for GR ($F_{2,29}$ = 4.09, P = 0.029; Fig. 3) and SOD ($F_{2,30}$ = 10.43, P = < 0.001). High salinity (9 ppt) resulted in an average reduction of 26.8 % in GR activity (P = 0.025) compared to control tadpoles, although tadpoles in 6 ppt did no vary GR activity. Tadpoles in 6 ppt reduced their SOD activity by 28.8 % (P = 0.005) compared with tadpoles in freshwater whereas the reduction in SOD activity at 9 ppt reached on average 35.9 %. We found no significant variation in CAT and GPx activity in response to salinity (all P > 0.686), and no evidence for oxidative cellular damage (TBARS; $F_{2,30}$ = 0.03, P = 0.962). Furthermore, salinity increased the proportion of neutrophils 41.3 % and 41.6 % on average in 6 ppt and 9ppt respectively ($F_{2,28}$ = 20.19, P < 0.001), whereas lymphocytes decreased by 10.1 % and 8.5 % in 6 ppt and 9 ppt, respectively ($F_{2,28}$ = 13.43, P < 0.001). The proportion of basophils or eosinophils did not vary among treatments (P > 0.286).

Responses to herbicide

Herbicide increased CORT levels ($F_{2,28}$ = 3.94, P = 0.033; Fig. 1) at both concentrations used, ranging from 65 % to 91.4 % increase, although in the case of the 2 mg/L glyphosate treatment the difference with the control was marginally non-significant (P = 0.060). Exposu-

re to herbicide also increased SMR ($F_{2,22}$ = 5.69, P = 0.0122; Fig. 2). SMR increased on average by 2.6-fold and 2.7-fold in 1 and 2 mg/L, respectively, both differing from the control treatment (P < 0.029). Herbicide exposure also altered antioxidant activity. GR activity decreased with herbicide ($F_{2,29}$ = 5.15, P = 0.012; Fig. 3) at both concentrations (37.6 % at 1 mg/L and 30.72 % at 2 mg/L) compared to tadpoles in non-herbicide treatment. GPx activity tended to decreased in tadpoles exposed to 1 mg/L of glyphosate, but this change was marginally non-significant ($F_{2,30}$ = 2.74, P = 0.082). We found no changes in SOD or CAT activity (P > 0.372), and no evidence of oxidative damage (TBARS; $F_{2,29}$ = 0.615, P = 0.548). Herbicide exposure did not significantly affect leukocyte proportion (P > 0.369).

Responses to changes in pH

Exposure to acid or basic pH did not cause tadpoles to vary their CORT levels ($F_{2,23}$ = 1.37, P = 0.278; Figure 1) or SMR ($F_{2,30}$ = 1.45, P = 0.448; P = 0.252; Figure 2). We found, however, a marginally non-significant decrease in GR activity ($F_{2,30}$ = 2.94, P = 0.070; Fig. 3) so that tadpoles exposed to pH 4.5 showed on average 20.5 % lower GR activity than tadpoles in neutral water. We found no significant changes in GPx, SOD, or CAT activities (all P > 0.205), and no sign of oxidative damage (TBARS; $F_{2,30}$ = 0.26, P = 0.774). Leukocyte count was similarly unaffected by exposure to acidic and basic pH (P > 0.267), except for the proportion of basophils which increased by 1.46-fold in tadpoles exposed to basic pH ($F_{2,24}$ = 4.76, P = 0.009).

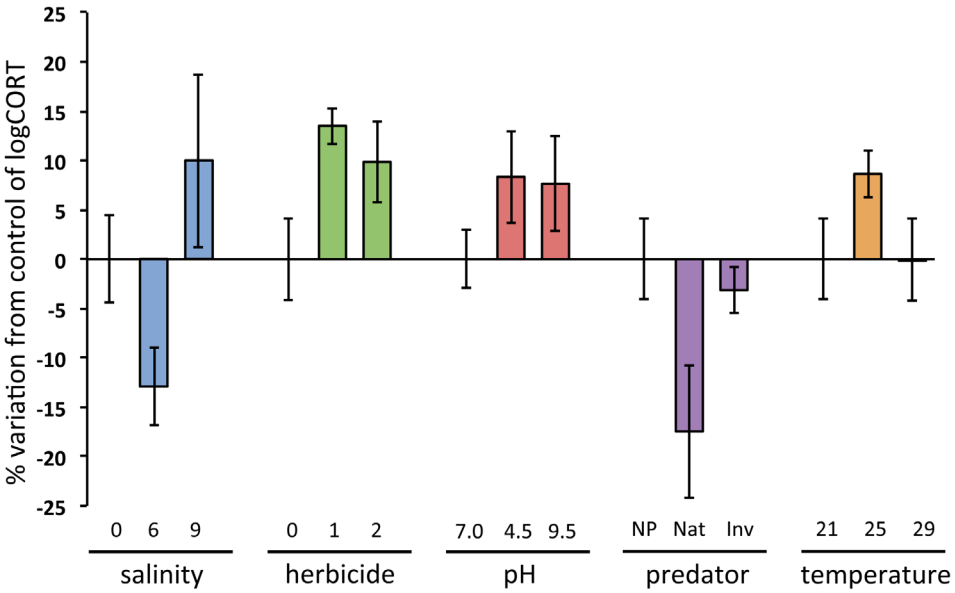

Figure 1. Effect of experimental stress factors on corticosterone concentration in spadefoot toad tadpoles, expressed as % variation from the average concentration (± s.e.) in control tadpoles. CORT was measured in pg/mL, after 10-days of exposure to each factor: salinity (0, 6, and 9 ppt), herbicide (0, 1, and 2 mg/L of glyphosate), pH (4.5, 7.0, and 9.5), predators (absence, native and invasive), and temperature (21 ºC, 25 ºC, and 29 ºC).

Responses to predator exposure

Exposure to predators altered CORT levels ($F_{2,23}$ = 4.11, P = 0.032; Figure 1). Tadpoles raised in the presence of native beetle larvae decreased CORT by an average of 50.1 % (P = 0.046). CORT levels, however, did not change when exposed to invasive crayfish (P = 0.618). Neither native nor alien predators altered SMR ($F_{2,29}$ = 0.37, P = 0.693; Figure 2). Likewise, the activity of antioxidant enzymes was unaffected by the presence of either type of predator (P > 0.081), and no cellular damage was detected ($F_{2,27}$ = 0.42, P = 0.66). Predators presence had no effect on leukocyte count (P > 0.275).

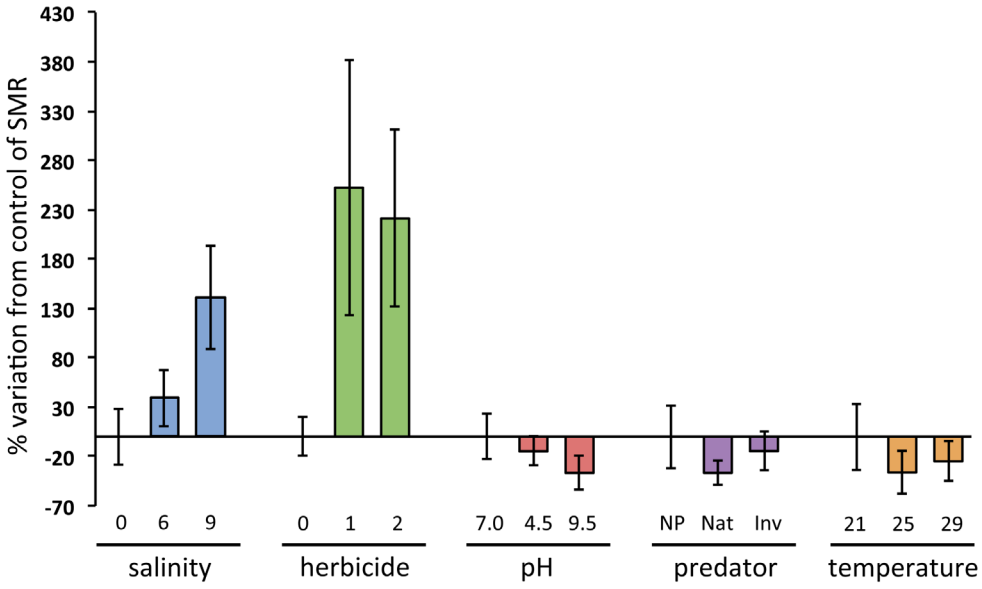

Figure 2. Percent deviation from average control values (± s.e.) of standard metabolic rate (SMR, in ml O_2/h/g) after 10-days of exposure to five factors: salinity (0, 6, and 9 ppt), herbicide (0, 1, and 2 mg/L of glyphosate), pH (4.5, 7.0, and 9.5), predators (absence, native and invasive), temperature (21 ºC, 25 ºC, and 29 ºC).

Responses to temperature

We observed no variation in CORT levels ($F_{2,26}$ = 1.83, P = 0.183; Figure 1) or SMR ($F_{2,27}$ = 0.80, P = 0.462; Figure 2) across the three temperatures used in the experiment. However, temperature affected the activity of antioxidant enzymes and leukocyte count. Tadpoles reared at 25 ºC decreased their SOD activity with respect to the other two temperatures (21 and 29 ºC; $F_{2,30}$ = 3.59, P = 0.041). GR activity also varied with temperature ($F_{2,30}$ = 5.80, P = 0.008; Figure 3), with tadpoles exposed to 25 ºC showing on average 30.6 % higher activity with respect to tadpoles reared at 21 ºC, and by 31.3 % with respect to those raised at 29 ºC. We found no effects of temperature on GPx or CAT activity (all P > 0.512). However, tadpoles exposed

to 25 ºC increased TBARS ($F_{2,30}$ = 4.98, P = 0.014) by 1.23-fold and 1.40-fold compared to tadpoles at 21 ºC and 29 ºC, respectively. Moreover, tadpoles in either 25 ºC or 29 ºC showed a decrease in the neutrophil proportion by 52.17 % and by 58.30 % and an increase in the lymphocyte proportion by 7.6 % and 8.4 % ($F_{2,18}$ = 15.12, P <0.001) compared to tadpoles raised at 21 ºC, respectively. The proportion of eosinophils decreased by 41.6 % and 85.3 % ($F_{2,18}$ = 3.63, P <0.026) in tadpoles exposed to 25 and 29 ºC, respectively. We did not find a significant change in basophil proportions ($F_{2,18}$ = 2.66, P < 0.070).

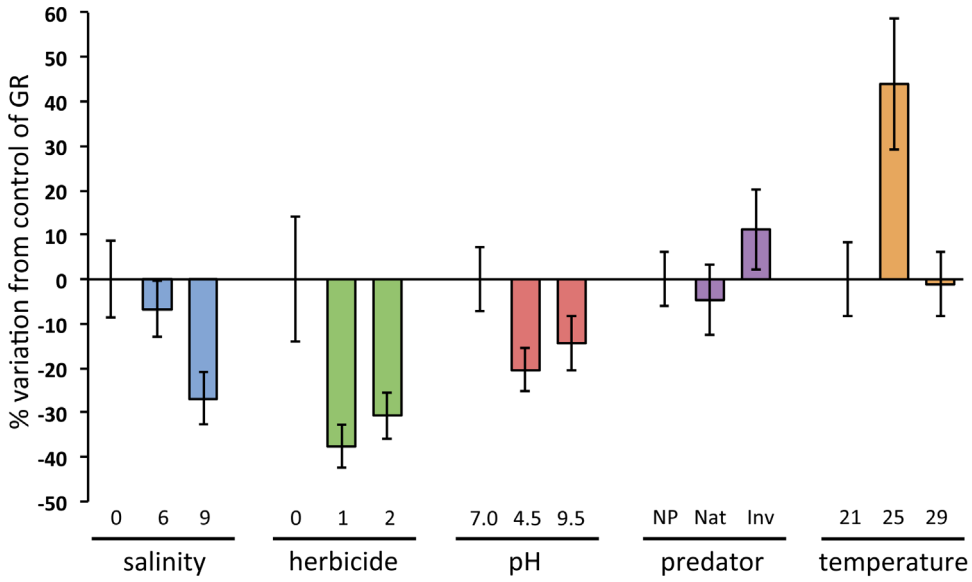

Figure 3. Percent deviation from control treatments (± s.e.) of glutathione reductase activity (GR, in U/mg prot) after 10-days of exposure against five stress factors: salinity (0, 6, and 9 ppt), herbicide (0, 1, and 2 mg/L of glyphosate), pH (4.5, 7.0, and 9.5), predators (absence, native and invasive), temperature (21 ºC, 25 ºC, and 29 ºC).

Discussion

Exposure to non-lethal levels of salinity, pH, temperature, herbicide (glyphosate), and predators caused marked physiological alterations in spadefoot toad tadpoles. Of all the potential stressors studied, salinity and herbicide seemed to impact amphibian physiology the most. These two factors altered CORT levels, standard metabolic rate, and antioxidant enzymes activity in *P. cultripes* tadpoles (Table 1). The highest levels of salinity and herbicide (i.e. 9ppt and 2 mg/L of glyphosate, respectively) produced comparable physiological unbalances since hormonal and metabolic rate changes occurred in the same direction and similar magnitude. These results outline that even non-lethal levels of stressors may have marked physiological effects, and is important to take that into consideration when designing conservation policies.

Both salinity and herbicide exposure caused tadpoles to increase their energy expenditure, possibly driven via increased corticotropin-releasing hormone that ultimately elevates thyroid hormone and CORT levels (Fig. 1) causing associated increases in metabolic rate (Denver et al. 2002, Wack et al. 2012; Fig. 2). Moreover, the activation of corticotropin-releasing hormone increases expression of mineralocorticoid receptors (Gesing et al. 2001). These receptors are involved in the regulation of body fluid osmolality and ion balance (Terker and Ellison, 2015), which is essential for amphibian osmoregulation, especially under osmotic stress (Hopkins and Brodie, 2015). As predicted, factors that increased CORT concentration and metabolic rate resulted in redox imbalance, as indicated by alterations in antioxidant enzyme activity (Fig. 3, Table 1). Decreased GR activity might be due to low levels of NADPH, a secondary manifestation of cellular free radical stress (Moreno et al. 2005). Likewise, decreased

SOD activity is likely related to oxidative inactivation of enzymes by free radicals (Pigeolet et al. 1990) due to reduced *de novo* synthesis (Kaushik and Kaur, 2003). Despite possible redox imbalance, no cellular damage in cell membranes was observed, as indicated by lack of differences among treatments in TBARS (Lin et al. 2004). In addition, salinity affected the leukocyte profile, causing increased proportions of lymphocytes and granulocytes (Table 1). Common stress-induced changes in the proportion of leukocytes include neutrophilia (abnormal high number of neutrophil) and lymphopenia (abnormal low number of neutrophil), are often associated with increased glucocorticoid levels (Davis et al. 2008). Herbicide, however, had no apparent effects on the immune system in this experiment, glyphosate has been shown to affect the leukocyte proportion of tadpoles (Shutler and Marcogliese, 2011; Burraco et al. 2013).

Contrary to our expectations, high temperature did not affect either CORT levels or SMR. This could be due to lack of statistical power, but other stressful factors did cause marked changes in these parameters, so at least we can conclude that the effect of temperature was milder than that of factors such as salinity or herbicide. Nevertheless, we observed increased GR activity at 25 ºC, as well as evidence for oxidative cellular damage (TBARS). Tadpoles at 25 ºC may have been closer to their optimum temperature (from a physiological point of view) and consequently experienced higher growth and developmental rates than tadpoles at either 21 ºC or 29 ºC, hence increasing lipid peroxidation (by-product of fat degradation). However, tadpoles reared at 29 ºC may have developed too quickly to even have time to accumulate fat (Kulkarni et al. 2011), hence reducing the rate of lipid peroxidation. More detailed analysis of lipid consumption during the course of anuran development and in response to changes in developmental and growth rates are needed to clarify the observed nonlinear patterns in oxidative stress with varying temperature.

Table 1. Averages ± standard errors of physiologycal parameters measured on tadpoles against five stressful factors: salinity, pH, predators, temperature and herbicide (glyphosate)

	CORT	SMR (lsmeans)	CAT	SOD	GPx	GR	TBARS	Gran %	Lymph %
Salinity 0 ppt	125.60 ± 27.51	0.040 ± 0.013	247.90 ± 14.70	142.90 ± 12.01	20.75 ± 1.03	7.18 ± 0.61	8.97 ± 1.34	23.66 ± 2.42	75.56 ± 2.42
Salinity 6 ppt	64.27 ± 9.74	0.047 ± 0.012	259.23 ± 16.79	106.14 ± 5.41	22.30 ± 1.56	6.53 ± 0.43	8.96 ± 1.08	31.33 ± 3.87	68.18 ± 3.87
Salinity 9 ppt	269.86 ± 118.38	0.095 ± 0.013	266.73 ± 14.88	91.58 ± 5.25	24.36 ± 2.13	5.25 ± 0.39	9.42 ± 1.81	30.15 ± 2.86	69.38 ± 2.86
Herbicide 0 mg/L	493.54 ± 144.73	0.010 ± 0.008	202.24 ± 9.70	107.50 ± 5.04	17.05 ± 2.66	13.60 ± 1.83	3.50 ± 1.11	17.41 ± 2.88	84.90 ± 3.84
Herbicide 1 mg/L	944.87 ± 104.78	0.032 ± 0.008	195.44 ± 7.54	120.91 ± 7.69	12.88 ± 1.50	8.48 ± 0.64	3.94 ± 0.84	17.53 ± 4.81	86.15 ± 1.89
Herbicide 2 mg/L	860.20 ± 147.53	0.028 ± 0.007	205.57 ± 7.64	119.18 ± 6.63	19.30 ± 1.83	9.43 ± 0.71	2.67 ± 0.56	18.68 ± 3.51	83.77 ± 2.27
pH 7.0	91.77 ± 10.78	0.061 ± 0.012	335.13 ± 34.79	153.62 ± 8.41	23.97 ± 2.25	8.26 ± 0.60	8.10 ± 0.79	13.85 ± 1.89	86.15 ± 1.89
pH 4.5	153.06 ± 35.95	0.052 ± 0.012	282.99 ± 13.18	134.70 ± 8.99	28.99 ± 3.23	6.57± 0.40	7.15 ± 0.74	16.64 ± 3.84	84.90 ± 3.84
pH 9.5	164.97 ± 40.89	0.040 ± 0.011	283.97 ± 20.19	132.70 ± 9.47	24.32 ± 2.09	7.07 ± 0.50	8.05 ± 1.48	15.19 ± 2.27	83.77 ± 2.27
No predator	168.84 ± 41.14	0.076 ± 0.019	217.40 ± 17.07	131.00 ± 5.80	22.84 ± 1.86	7.56 ± 0.45	8.02 ± 0.69	18.44 ± 2.94	83.96 ± 3.22
Native	83. 78 ± 24.42	0.048 ± 0.017	307.14 ± 40.65	117.16 ± 14.08	23.62 ± 1.90	7.21 ± 0.59	7.57 ± 1.28	16.3 ± 2.42	92.21 ± 4.00
Invasive	131.92 ± 13.43	0.064 ± 0.017	294.03 ± 40.49	146.41 ± 16.76	23.82 ± 1.49	8.41 ± 0.64	6.76 ± 1.03	15.90 ± 2.60	90.95 ± 3.77
21 ºC	444.51 ± 69.29	0.024 ± 0.006	171.20 ± 4.04	105.06 ± 2.14	16.62 ± 1.66	8.42 ± 0.69	1.64 ± 0.49	16.04 ± 3.22	81.56 ± 2.94
25 ºC	672.06 ± 93.88	0.015 ± 0.007	173.46 ± 8.03	94.75 ± 6.45	17.95 ± 1.75	12.12 ± 1.24	3.66 ± 0.72	7.79 ± 4.00	83.67 ± 2.42
29 ºC	460.33 ± 102.10	0.018 ± 0.006	170.45 ± 7.18	110.88 ± 3.08	14.54 ± 2.66	8.32 ± 0.60	1.52 ± 0.35	9.05 ± 3.77	84.11 ± 2.60

The physiological parameters included in this table are: corticosterone (CORT, in pg/ml), lsmeans of standard metabolic rate (SMR, in ml O_2/h/g), catalase (CAT, in U/mg prot), superoxide dismutase (SOD, in U/mg prot), glutathione peroxidase (GPx, in mU/mg prot), and glutathione reductase (GR, in U/mg prot) activity, thiobarbituric acid reactive substances (TBARS, in nmol/ml), and granulocyte (Gran) and lymphocyte (Lymph) percentage (%) of total of white cells count.

Changes in pH within the range used in this study did not cause deep physiological adjustments. A slight (and marginally non-significant) reduction in GR activity may reflect increased free radicals

against acidic and basic pH. Chambers and Belden (2009) found effects of acid or basic pH on CORT levels in amphibian larvae but theses changes were species-dependent. The range of water pH in the Doñana National Park is wide (from 4-5 to 7-8; Serrano et al. 2006) thus *P. cultripes* tadpoles could be adapted to large fluctuations of pH.

The observed responses to predators indicate a conflict between the population-level and the organismal-level concepts of environmental stress. Predators clearly pose a threat to individual survival and could consequently dramatically reduce fitness within populations. In that respect, predators are clearly a source of environmental stress. In some species, predators also trigger physiological stress responses that would typically characterize them as a stressful factor, like when raptors induce overexpression of heat-shock proteins in nestling passerines (Thomson et al. 2010). Similarly, Maher et al. (2013) reported CORT elevation in *Rana sylvatica* tadpoles exposed to dragonfly nymphs. However, we observed reduced CORT levels in spadefoot toad tadpoles exposed to native beetle larvae (Fig. 1). Reduced CORT in the presence of predators may simply be associated to the reduction in activity rate observed in *P. cultripes*, which can be up to 57 % in the presence of native predators (Polo-Cavia and Gomez-Mestre, 2014). Other amphibians have been shown to also lower their metabolic rate in the presence of predators (Barry and Syal, 2013), sometimes after an initial transient increase (Steiner and Van Buskirk, 2009). Invasive predators, however, did not alter tadpoles' CORT levels (Fig. 1). This lack of hormonal response to invasive crayfish is congruent with past observations that local tadpoles do not activate their morphological or behavioural defences against invasive crayfish at the study site for lack of innate recognition (Gomez-Mestre and Díaz-Paniagua, 2011; Polo-Cavia and Gomez-Mestre, 2014).

Our data show that common stressors to aquatic systems substan-

tially alter the physiological state of tadpoles. Herbicides constitute a major threat because they are novel to amphibians at an evolutionary scale and have marked physiological consequences. However, while most of the other factors may vary considerably in nature, they are often drastically intensified by human activities, as in salinization or acidification of aquatic systems, raises in temperature, or the introduction of alien predators. In particular, high levels of salinity and herbicide cause similarly steep physiological alterations in tadpoles. It is however important to understand the nature of the responses against each type of stressor, because different risks may induce physiological changes of very different magnitude and even in opposite directions. Among the parameters used, CORT and GR were the most sensitive to environmental stress in our study, although a combined approach determining several other physiological parameters such as metabolic rate or leukocyte profile provides a more comprehensive assessment of the physiological responses. Systematic comparisons of physiological alterations against multiple factors and factor combinations will fuel larger scale comparative physiology, providing mechanistic insights into conservation, ecological, and evolutionary studies, and contributing to explain large geographical and temporal patterns (Chown and Gaston, 2015). Moreover, stress experienced during early life stages and high levels of glucocorticoids in particular have long-lasting effects (Weaver 2009, Wu et al. 2012). Therefore, long-term studies are needed to fully understand the consequences of stress during the larval stages on the phenotype and fitness of the adults. Comparative physiological studies will also contribute to inform effective management decisions aimed at soothing the impact of anthropogenic disturbances before marked population declines are detected (Chown and Gaston, 2008).

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CHAPTER 2

Different effects of accelerated development and enhanced growth on oxidative stress and telomere shortening in amphibian larvae

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Abstract

Organisms react to environmental changes through plastic responses that often involve physiological alterations with the potential to modify life-history traits and fitness. Environmentally induced shifts in growth and development in species with complex life cycles determine the timing of transitions between subsequent life stages, as well as body condition at transformation, which greatly condition survival at later stages. Here we show that alterations in growth and development in spadefoot toad larvae markedly affect their fat reserves, oxidative stress, and relative telomere length. Tadpoles accelerated development but reduced growth and consumed more fat reserves when facing pond drying. However, oxidative stress due to developmental acceleration was buffered by increased antioxidant enzyme activity, and telomeres remained unchanged. Predators caused opposite effects: they reduced larval density, hence relaxing competition and allowing faster development and enhanced growth of survivors. Tadpoles surviving predators metamorphosed bigger and had larger fat bodies, increasing their short-term survival odds, but showed signs of oxidative stress and shortened telomeres. Developmental acceleration and enhanced growth thus had different physiological consequences: reduced fat bodies and body size compromise

short-term survival, but are reversible in the long run, whereas telomere shortening due to rapid growth is non-reversible and will reduce long-term survival.

Keywords: Amphibians; Developmental plasticity; Enhanced growth; Fat reserves; Oxidative stress; Pond drying; Predators; Telomere shortening

Introduction

Selection in heterogeneous environments often results in the evolution of adaptive phenotypic plasticity (Sultand and Spencer, 2002; Gomez-Mestre and Jovani, 2013). However, phenotypic alteration is often costly, costs referred to as 'production costs' (Auld et al. 2010). Known production costs involve physiological alterations such as immune state suppression or increased metabolism, which result in changes in life-history traits and ultimately affect fitness4. Understanding the physiological effects of plastic alterations of the phenotype will help us assess their short-term and long-term consequences (Ricklefs and Wikelski, 2002; Monaghan et al. 2009).

Stress responses to environmental challenges in vertebrates are orchestrated via activation of neuroendocrine pathways of which the hypothalamic-pituitary-adrenal (HPA) axis is perhaps the most studied. HPA-axis activation induces the mobilization of metabolites (Remage-Healey and Romero, 2001) but may result in immunological imbalances (Sapolsky et al. 2000) that produce profound alterations in developmental and growth rates (Wingfield et al. 1998; Denver, 2009). Such environmentally induced alterations of growth and development are particularly relevant in species with complex life cycles (Metcalfe and Monaghan, 2001) because the timing of transitions between life stages and body condition at transformation

greatly determine survival at later stages (Pechenik, 2006). Accelerating or slowing development and growth have marked consequences across most taxa, ranging from changes in protein turnover within tissues (Samuels and Baracos, 1995), to allometric changes in body shape and degree of ossification (Gomez-Mestre et al. 2010), to variation in fat storage (Kulkarni et al. 2011). From a molecular perspective, enhanced developmental and growth rates may result in two major physiological alterations with extensive consequences for fitness: telomere shortening and oxidative stress (Bize et al. 2009; Heidinger et al. 2012).

Telomeres are non-coding repetitive terminal regions of the chromosomes specialized in chromosome protection from deterioration, or from fusion with other chromosomes (Capper et al. 2007). Telomere sequences are restored via reverse transcriptase telomerase that adds telomeric repeats (TTAGGG in vertebrates) to 3' overhang. Critical telomere shortness stops cell division and initiates a state of replicative senescence leading to programmed cell death (Campisi et al. 2007). Telomere shortening is thought of as an internal clock that could potentially be used for estimating chronological age in the wild, although telomere length can vary at different rates over different ontogenetic stages across species. Patterns of telomere length variation over time can be rather complex, as for example in mice, where telomeres can even elongate during very early embryonic development (Liu et al. 2007), much like in human stem cells, B cells or some tumor cells (Weng et al. 1997; Henson et al. 2002; Reddel, 2003). Therefore, spontaneous ontogenetic shifts from telomere elongation to shortening, combined with physiological alterations of the rate of telomere shortening loosen the link between biological and chronological ageing (Bize et al. 2009; Heidinger et al. 2012). Interestingly, the fact that telomere shortening is susceptible of alteration due to phy-

siological adjustments of the organisms to the environmental conditions experienced, provides the means to evaluate the relative costs and trade-offs of phenotypic responses to environmental challenges, especially in early ontogenetic stages (Heidinger et al. 2012, Herborn et al. 2014).

Developmental acceleration and enhanced growth have been shown to cause telomere shortening as observed *in vitro* in rat pancreatic islets (Tarry-Adkins et al. 2009) probably as a consequence of multiple cell divisions, a phenomenon referred to as the 'end replication problem' (Jennings et al. 2000), and of oxidative damage accumulated over the cellular lifespan (Von Zglinicki, 2002; Geiger et al. 2012). Accelerated development and enhanced growth, as well as acute episodes of environmental stress, produce excess reactive oxygen substances (ROS) that can result in severe oxidative stress and damage cell structures such as telomere sequences (Monaghan et al. 2009). Both reduced telomere length and increased oxidative stress seem to play a key role in ageing and are good predictors of individual lifespan (Monaghan et al. 2009; Shalev et al. 2013) although further evidences are still needed (Simons, 2015).

Most systems studied so far in the context of the interplay of oxidative stress and telomere shortening as a consequence of environmentally induced phenotypic responses have focused on taxa in which growth and development tended to be rather correlated, such as mammals (mainly humans; Von Zglinicki, 2002) and birds (Horn et al. 2010). However, amphibian larvae have been much less studied in this context despite being an ideal system for evaluating the consequences of developmental and growth plasticity separately. The development of most amphibian species include abrupt ontogenetic switch points in which timing is usually highly plastic since larvae readily modify their activity, morphology, differentiation rate, and

growth rate in response to environmental cues (Van Buskirk, 2002; Hua et al. 2015). Two main environmental hazards for amphibian larvae are predators and pond drying (Skelly, 1997). Tadpoles are capable of detecting and responding plastically to both risks. However, responses against pond drying and predators seem to be opposite in many respects. Thus, amphibian larvae accelerate development and decrease growth under pond drying conditions (Ritchter-Boix et al. 2011). In contrast, predators induce reduced activity and metabolism of amphibian larvae (Steiner and Van Burskirk, 2009). In addition, predators directly reduce larval density hence relaxing competition and allowing the surviving larvae to reach metamorphosis faster and at a larger size (Relyea, 2007). Analysing the physiological consequences of changes in growth and development in amphibian larvae is important to understand both short-term and long-term carry-over effects of adaptive plastic responses.

Here we evaluate the effects of altered developmental and growth rates in western spadefoot toad tadpoles (*Pelobates cultripes*) as a consequence of pond drying and presence of freely roaming predators. We examined the physiological consequences of such alterations in development and growth on the surviving larvae of each treatment in terms of their fat body content, oxidative stress, and telomere length after metamorphosis. We expected tadpoles to accelerate development in response to pond drying, but at the expense of metamorphosing at a smaller size and with reduced fat reserves. We also hypothesized that pond drying would involve telomere shortening and oxidative damage as a consequence of the increased metabolic effort required for developmental acceleration. Similarly, we expected reduced larval density due to predation to result in lower competition, hence providing better growing conditions for the surviving larvae. Therefore, we expected individuals surviving predators to

have a larger mass at metamorphosis, and more abundant fat reserves. In terms of oxidative stress, high resource availability could entail increased metabolism and ROS production, which would have to be balanced with increased antioxidant enzyme activity. Fast growing individuals would be expected to have undergone a greater number of rounds of cell replication, resulting in shortened telomeres.

Material and methods

Bioethics and Animal Care

All experimental procedures in our study were evaluated and approved and euthanasia of juvenile toads were conducted at Estación Biológica de Doñana, CISC, following protocol '12_53-Gomez' approved by the Institutional Animal Care and Use Committee (IACUC) at Estación Biológica de Doñana. All experiments were performed in accordance with relevant guidelines and regulations at the national and European levels.

Field sampling

In March 2014, we collected between 60-70 eggs from each of ten *Pelobates cultripes* clutches from two different locations in Southern Spain: five clutches from Sierra Norte Natural Park (Seville province) and five from Doñana National Park (Huelva province). While our aim was never to compare across sites, we included in the study clutches from two different areas to ensure that we had substantial variation among families. *Pelobates cultripes* larvae are the biggest amphibian larvae in the Iberian Peninsula, have a long larval period (typically 4-6 months), and are commonly exposed to risk of predation and

pond drying. However, *P. cultripes* can plastically modify their rates of growth and development (Kulkarni et al. 2011) by altering corticosterone and thyroid hormone concentrations, and upregulating expression of hormone receptors (Gomez-Mestre et al. 2013; Burraco et al. 2016).

Individuals from each clutch (*family*) were raised from eggs for two months prior to the beginning of the experiment. During this period, larvae from each family were raised outdoors separately in 500 L mesocosms (100 cm in height and 120 cm in the upper diameter) filled with 400 L of well water and fed *ad libitum* with natural aquatic plants (*Ranunculus peltatus, Myriophyllum alterniflorum*, and *Callitriche obtusangula*), supplemented with rabbit chow. To avoid crowding effects during this initial period we randomly put only 30 tadpoles from each family in each of two tanks. We also collected aquatic beetle larvae (*Dytiscus circumflexus*) in natural ponds and kept them until the onset of the experiment. *Dytiscus* larvae are common and very effective predators of amphibian larvae in the study area.

Experimental setup

The experiment was conducted in similar tanks as the ones used to initially raise tadpoles. We prepared the tanks for the experiment by adding 130 kg of sand (20-cm deep layer) to each tank plus 5 kg of dry sediment from the basin of several temporary ponds in Doñana National Park to provide a substrate for macrophytes to root in (Arribas et al. 2014). We then filled each tank with 400L of well water and allowed macrophytes, zooplankton, and phytoplankton to grow naturally. Tanks were covered with fiberglass window screen to avoid insect colonization. Two months after egg hatching we introduced the tadpoles in the experimental tanks to initiate the experiment. Tadpoles were between Gosner stages 27 and 30 (Gosner, 1960) at the

onset of the experiment. We crossed two levels of the factor water regime (constant high water level or simulated pond drying) with the presence or absence of predators in a 2x2 experimental design. In each tank we put 4 tadpoles from each family for a total of 40 tadpoles per tank. This larval density is well within the range commonly observed in the field (Arribas et al. 2014). Each treatment was replicated 6 times for a total of 24 tanks. In order to keep track of the different families once mixed in each tank and to be able to control for possible family effects on physiological measurements, we marked all tadpoles with Visible Implant Elastomer (VIE) tags (Northwest Marine Technology, Inc.). VIE tags were introduced subcutaneously in the dorsal part of the body with a 29-G insulin syringe (BD Micro-Fine Insuline U-100 0.5 ml). We used 5 different colours (yellow, red, pink, green and blue) of VIE tags that were put in the back or the front of the head, therefore using 10 different combinations of VIE tags to distinguish the 10 families used in the experiment. VIE tags and positions (anterior or posterior position along the body) were randomized among families and tanks to avoid possible sensory biases from predators towards specific VIE tags that could have resulted in biased predation risk with respect to family identity. That way, different families were tagged with randomly assigned colours and positions in each experimental tank. Marked tadpoles were monitored during 24 hours in 10-L tanks after VIE tags were introduced. All tadpoles survived 24-hours after VIE tag implantation.

One predator was introduced in each tank randomly assigned to the predator presence treatments. Predators (dytiscid water beetles) were first placed in cages for two days using lidded 1-L plastic buckets left afloat and with small holes drilled at the bottom. Caged predators hence provided water borne chemical cues and allowed tadpoles to build up behavioural anti-predatory responses, which are

activated immediately upon exposure to predator cues. On the third day, we released the *Dytiscus* larvae inside each tank, allowing them to prey on tadpoles for only 14 days to avoid prey depletion. After that period, predators were caged again in the same floating cages until all surviving tadpoles had metamorphosed. Tanks assigned to predator absence contained empty floating cages. In the tanks assigned to simulated pond drying we removed 30 L of water twice weekly until only a column 10 cm high remained (approximately 50 days after the onset of the experiment), thereafter keeping the volume constant until the end of the experiment. In those tanks assigned to constant water level we added water weekly to keep a constant water level of 80 cm high (400 L).

Tanks were surveyed every other day to retrieve metamorphosing individuals (i.e. individuals at forelimb emergence, Gosner stage 42). Metamorphs were individually maintained in 500 mL lidded cups with 2 mm of pond water at the bottom until they completed tail resorption (Gosner stage 46). Then, they were blotted dry and weighed to the nearest 0.0001 g. We photographed all juveniles and used the images to estimate snout-to-vent length (SVL) using Image J 1.46r (NIH, USA). Finally, juveniles were euthanised by immersion in a lethal concentration of MS-222, then snap frozen in liquid nitrogen and stored at -80 ºC. For fat storage, antioxidant enzymes activity and relative telomere length determination we randomly selected the same subset of 33 marked juveniles from each treatment, except for the combined pond drying plus predator treatment for which we only retrieved 11 marked juveniles recovered at the end of the experiment. Therefore, oxidative stress and relative telomere length measurements were conducted in the same individuals, although in different parts of the body. For oxidative stress assays we used whole eviscerated individuals because the amount of tissue required for

assaying the different enzymes was elevated. Instead, for relative telomere length assays we used only a small portion of the leg muscle.

Fat bodies

Randomly selected juveniles were thawed and dissected for determination of weight of fat bodies on an analytical balance with resolution to the nearest 0.0001 g (CP324S, Sartorius).

Oxidative stress

We determined the activity of four antioxidant enzymes: catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR). We also measured malondialdehyde (MDA) formed during lipid peroxidation, and the oxidized and total reduced glutathione (GSSG and GSHt, respectively), as indicators of oxidative cell damage. Tadpoles were eviscerated to avoid possible interferences of the intestinal content and were immersed in a buffered solution to inhibit proteolysis (100 mM Tris-HCl with 0.1 mM EDTA, 0.1 % triton X-100, pH 7.8 and 0.1 mM PMSF; Burraco et al. 2013) and were homogenized at 35,000 rpm with a Miccra homogenizer (Miccra D-1). We used a proportion of 1 g of homogenized tadpole in 4 mL of homogenization buffer (1:4, w:v). We centrifuged the homogenates at 20817 g for 30 min at 4º C and aliquoted supernatants into several 0.6 mL tubes and stored at -80 ºC. We determined total protein content and we quantified CAT, SOD, GPx, GR, and MDA according to standard methods (for details and references see Additional File 1) as well as GSH_t levels and the ratio GSH/GSSG (see Additional File 1).

Relative telomere length

We extracted genomic DNA for relative telomere length determination from leg muscle, using a commercial kit for genomic DNA isolation (QIAGEN DNAeasy Blood&Tissue Kit). Genomic DNA was stored at -20 ºC until assayed. We measured relative telomere length from a single tissue (leg muscle) to avoid possible differences in this measurement among cell types (Miyashita et al. 2002).

Relative telomere length assays were performed using quantitative PCR (qPCR). This is an efficient and high-throughput method for measuring telomeric repeats in vertebrates despite potential confounding amplification in some species of interstitial TTAGGG sequences located outside of telomere regions (Nussey et al. 2014). We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control, whose forward and reverse primers sequences were 5-AACCAGCCAAGTACGATGACAT-3' (GAPDH-F) and 5'-CCATCAGCAGCAGCCTTCA-3' (GAPDH-R), respectively. Forward and reverse of the target gene was 5'CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3' (Tel1b) and 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3' (Tel2b), respectively. We performed qPCR for GAPDH and telomere genes on two separated plates using 20 ng of genomic DNA from each sample. The combined set of primers (Tel1b/Tel2b and GAPDH-F/GAPDH-R) was used at a concentration of 900 nM/900nM in a final volume of 25 µL containing 12.5 µL of Brilliant SYBR Green QPCR Master Mix (Stratagene; Bize et al. 2009). PCR cycles for telomere fragment amplification consisted in 10 min at 95 ºC followed by 30 cycles of 1 min at 56 ºC and 1 min at 95 ºC whereas for GAPDH fragment were 10 min at 95 ºC followed by 40 cycles of 1 min at 60 ºC and 1 min at 95 ºC. All qPCRs were carried out on LightCycler 480 (Roche). We tested the efficiency of each qPCR pla-

te performing a standard curve by serially diluting a pool of samples from the different treatments (160, 40, 10, 2.5 and 0.66 ng of DNA per well) in triplicate. We calculated the Cycle threshold (Ct) value of the reference sample for each plate. We run all experimental samples in duplicate and we used the mean values to calculate the relative T/S ratios, where T is the telomere repeat copy number and S is the single control gene (GAPDH) copy number, by applying the following formula (Pflaffl, 2001):

T/S ratio = $[(E_{telomere})^{\Delta Ct\ telomere\ (control - sample)}] / [(E_{GAPDH})^{\Delta Ct\ GAPDH\ (control - sample)}]$

where $E_{telomere}$ is the real-time PCR efficiency of telomere portion; E_{GAPDH} is the real-time PCR efficiency of the GAPDH portion; ΔCt telomere is the Ct deviation of control – sample of the telomere portion; ΔCt GADPH is the Ct deviation of control – sample of reference of GADPH (gene of reference) portion. We also estimated the specificity of the melting curve to check for possible primer dimers or secondary amplifications, and we found no indication of undesirable amplifications. The repeatability was 95 % for the GAPDH assay (i.e. 5 % error rate) and 94 % for the telomere assay. The average among-plates efficiency was 2.07 ± 0.052 and 1.87 ± 0.014 for GAPDH and telomere plates, respectively. The average within-plate coefficient of variation was 1.09 % and 0.97 % for GADPH and telomere assays, respectively. The among-plate coefficient of variation was 7.8 % for GAPDH and 10.8 % for telomere plates.

Statistical analyses

All statistical tests were conducted in R (R Development Core Team 2014, version 3.0.2). We observed the distribution of residuals and then tested for normality with a Kolgomorov-Smirnov test (lillie.test in package “nortest”, version 1.0-2). We also tested for homoscedas-

ticity of the data using a Barlett's test (bartlett.test function in "car" package, version 2.0-22). We fitted linear and generalized mixed models to include both fixed and random effects. We run "lmer" (for parametric data) and "glmer" (for non-parametric data) functions using the package "*lme4*" (version 1.1-7). The variables *tank* and *family* were introduced in the models as random factors. We also tested the significance of the variable *location* in all models but it was non-significant and we excluded it from the models. In all models, we used likelihood ratio tests to determine the significance of each factor. Normally distributed data (body length, days to metamorphosis, GR, GSSG, GSHt, and telomere data) were modelled with a Gaussian error distribution. Non-normally distributed data (body mass, growth, fat bodies, CAT, GPx, SOD, and MDA) were modelled with a Gamma distribution except for the analysis of survival, where we used a binomial distribution. We tested the effect of treatments on growth as log(body mass) – log(larval period) and relative fat body content as log(fat body mass) – log(body mass). Relative telomere length and GR values were log-transformed to fit parametric assumptions. Full statistical results can be found in the Additional File 2.

Results

Survival

During the experiment 324 individuals survived and completed metamorphosis out of the initial 960. Of these, 79 % kept color markings (VIE tags, see Methods) when they reached metamorphic climax (Gosner stage 42) and could therefore be assigned to sibship. Pond drying significantly reduced tadpole survival by 41.88 % (df = 1, 959; χ^2 = 12.005, $P < 0.001$; Figure 1a), whereas predators

reduced it by 72.41 % (df = 1, 959; χ^2 = 30.331, P < 0.001; Figure 1a). However, we did not find a significant interaction between pond drying and predator presence in tadpole survival (df = 1, 959; χ^2 = 0.241, P = 0.623; Figure 1a).

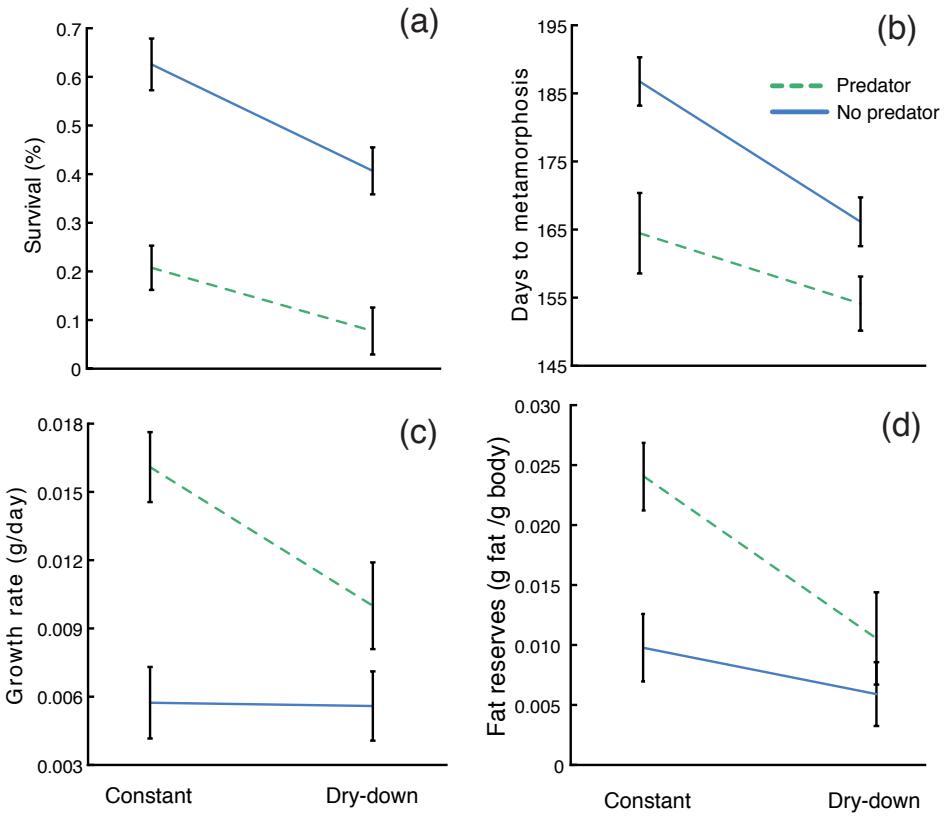

Figure 1. The effect of pond drying and predators on (a) survival, (b) larval period (days to metamorphosis), (c) growth rate, and (d) fat reserves in *Pelobates cultripes* metamorphosing from different larval conditions. Data are least square means ± standard error. The water level regime is indicated as 'Constant' and 'Dry-down' and the lines indicate the presence (P, green dashed line) or absence (NP, blue solid line) of predators.

Time to and size at metamorphosis

Pond drying resulted in shorter developmental period of the surviving larvae, as they reached metamorphosis on average 11.25 days earlier than in constant water (df = 1, 323; χ^2 = 15.265, $P < 0.001$; Figure 1b). This represents an average developmental acceleration of 18.72 %. The presence of freely roaming predators also resulted in shorter times to metamorphosis for surviving larvae (df = 1, 323; χ^2 = 17.713, $P < 0.001$; Figure 1b), showing an average reduction of 14.01 days to metamorphosis with respect to tadpoles in the absence of predators (22.88 % shorter larval periods on average). Again, we did not find a significant interaction between both factors in duration of the larval period (df = 1, 323; χ^2 = 0.990, $P = 0.320$; Figure 1b).

Size at metamorphosis of surviving individuals was also greatly affected by both pond drying and predators (Table 1), but in opposite directions. Pond drying caused an average reduction in body mass at metamorphosis of 41.85 % (df = 1, 279; χ^2 = 10.653, $P = 0.001$), whereas direct predation resulted in 58.71 % heavier body mass at metamorphosis of the surviving individuals (df = 1, 279; χ^2 = 24.451, $P < 0.001$). The interaction between pond drying and predator presence was not significant for body mass at metamorphosis (df = 1, 279; χ^2 = 0.0798, $P = 0.778$). In terms of body length (snout-to-vent length), pond drying resulted in 12.37 % shorter toadlets (df = 1, 267; χ^2 = 6.539, $P = 0.011$; Table 1), whereas predator presence resulted in 20.97 % longer ones on average (df = 1, 267; χ^2 = 23.755, $P < 0.001$; Table 1). The interaction between both factors was not significant for body length (df = 1, 267; χ^2 = 1.102, $P = 0.2940$). Growth rate of individuals surviving pond drying was reduced by 10.05 % on average (df = 1, 279; χ^2 = 6.009, $P = 0.014$; Figure 1c), but increased in larvae surviving predators by 18.49% on average (df = 1, 279; χ^2 = 28.586,

$P < 0.001$; Figure 1c). The interaction between both factors was significant ($\chi^2 = 2146.5$, $P < 0.001$; Figure 1c).

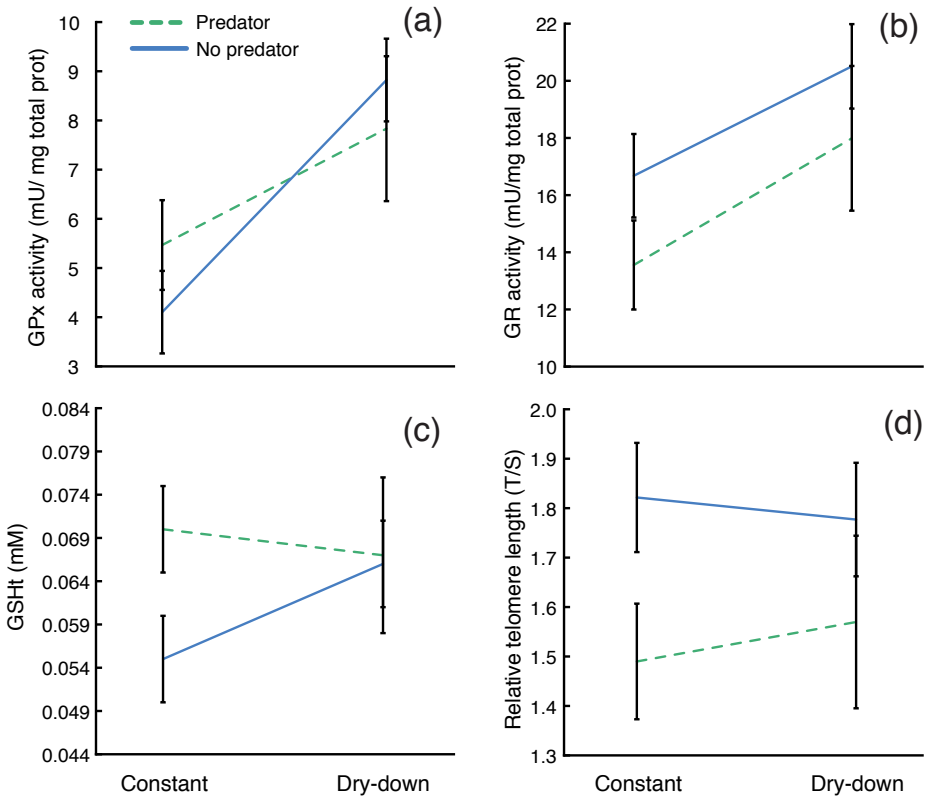

Figure 2. The effect of pond drying and predators on (a) glutathione peroxidase (GPx) activity, (b) glutathione reductase (GR) activity, (c) Total reduced glutathione (GSHt) level, and (d) relative telomere length (T/S) in surviving juveniles of *Pelobates cultripes*. Data are least square means ± S.E. The water level regime is indicated as 'Constant' and 'Dry-down' and the lines indicate the presence (P, green dashed line) or absence (NP, blue solid line) of predators.

Table 1. Least square means ± standard errors of measurements obtained in surviving spadefoot toad juveniles (*Pelobates cultripes*) exposed to four environmental conditions in 500-L tanks. The variables measured were: survival (%), days to metamorphosis (days to reach 46 Gosner stage from the egg), growth rate (g/day of development), snout-to-vent length (SNV; mm), and body mass (g). Fat storage was also measured (g). Moreover, we measured the following physiological parameters: catalase activity (CAT; U/mg total protein), glutathione peroxidase activity (GPx; mU/mg total protein), glutathione reductase activity (GR; mU/mg total protein), superoxide dismutase activity (SOD; U/mg of total protein), malondialdehyde (MDA; nmol/ml), reduced glutathione (GSH; mM), the ratio of reduced to oxidized glutathione (GSH/GSSG), and the relative telomere length (T/S ratio).

Life-history traits and fat storage

	Survival	Days to met.	Growth rate	SNV	Body mass	Fat storage
High water - No predator	62.22 ± 5.54	186.74 ± 3.55	0.0062 ± 0.0010	21.35 ± 0.90	1.14 ± 0.16	0.0098 ± 0.0028
High water - Predator	21.01 ± 4.82	164.46 ± 5.91	0.0153 ± 0.0009	27.20 ± 0.91	2.47 ± 0.15	0.0240 ± 0.0027
Low water - No predator	40.73 ± 5.14	166.14 ± 3.58	0.0055 ± 0.0009	20.17 ± 0.87	0.89 ± 0.15	0.0059 ± 0.0027
Low water - Predator	7.86 ± 5.14	154.12 ± 3.98	0.011 ± 0.0014	24.01 ± 1.10	1.65 ± 0.21	0.0105 ± 0.0038

Oxidative stress and relative telomere length

	CAT	GPx	GR	SOD	MDA	GSHt	GSH/GSSG	T/S
High water - No predator	74.54 ± 7.45	4.10 ± 0.84	16.68 ± 1.46	22.80 ± 1.36	16.01 ± 2.03	0.051 ± 0.005	5.03 ± 0.33	1.82 ± 0.11
High water - Predator	49.03 ± 7.24	5.47 ± 0.91	13.56 ± 1.56	21.73 ± 1.39	18.72 ± 2.08	0.066 ± 0.005	4.38 ± 0.33	1.49 ± 0.12
Low water - No predator	68.17 ± 7.31	8.83 ± 0.84	20.57 ± 1.48	26.18 ± 1.39	13.51 ± 2.06	0.062 ± 0.005	4.24 ± 0.33	1.78 ± 0.11
Low water - Predator	68.72 ± 11.99	7.83 ± 1.47	17.99 ± 2.53	24.13 ± 2.47	20.40 ± 3.39	0.063 ± 0.009	3.93 ± 0.63	1.57 ± 0.17

Fat bodies

Fat body content was reduced by an average of 19.79 % in juveniles that experienced pond drying during their larval development (df = 1, 108; $\chi^2 = 6.166$, $P = 0.013$; Figure 1d). In turn, fat bodies were on average 18.21 % heavier in juveniles surviving predators than in those metamorphosing from predator free tanks (df = 1, 108; $\chi^2 = 3.886$, $P = 0.049$; Figure 1d). The interaction between both factors was not significant (df = 1, 108; $\chi^2 = 1.944$, $P = 0.163$; Figure 1d).

Oxidative stress and relative telomere length

We found several alterations in the activity of antioxidant enzymes and in oxidative damage in the surviving individuals across experimental factors (Table 1). Neither pond drying nor predation altered CAT activity (df = 1, 104; χ^2 = 0.302, *P* = 0.582; df = 1, 104; χ^2 = 1.707, *P* = 0.191, respectively). Conversely, individuals surviving pond drying increased their GPx activity by an average of 44.13 % (df = 1, 99; χ^2 = 9.54, *P* = 0.002; Figure 2a) whereas individuals surviving predators did not (df = 1, 99; χ^2 = 0.461, *P* = 0.497; Figure 2a). Furthermore, pond drying increased GR activity by 22.20 % (df = 1, 104; χ^2 = 6.647, *P* = 0.010; Figure 2b) whereas predators reduced it by 20.91 % (df = 1, 104; χ^2 = 7.630, *P* = 0.006; Figure 2b). Finally, SOD activity remained unaltered in individuals surviving either pond drying (df = 1, 104; χ^2 = 2.297, *P* = 0.130) or predators (df = 1, 104; χ^2 = 0.907, *P* = 0.341). Alterations in the activity of antioxidant enzymes induced by pond drying did not entail changes in lipid peroxidation as indicated by the absence of variation in MDA concentration (df = 1, 103; χ^2 = 0.084, *P* = 0.772). Individuals that survived predators showed a non-significant decrease of MDA concentration (df = 1, 103; χ^2 = 3.504, *P* = 0.061). Total reduced glutathione (GSHt) was 13.47 % higher in individuals surviving predators (df = 1, 99; χ^2 = 4.468, *P* = 0.034; Figure 2c) than in those from tanks without predators. However, GSH remained unaltered in individuals surviving pond drying (df = 1, 99; χ^2 = 2.032, *P* = 0.154; Figure 2c). Individuals surviving pond drying experienced a marginally non-significant increase in the GSH/GSSG ratio (df = 1, 99; χ^2 = 3.499, *P* = 0.061), whereas those that survived predators did not alter their GSH/GSSG ratio at all (df = 1, 99; χ^2 = 2.168, *P* = 0.140; Table 1). We found no significant interactions between both factors in terms of antioxidant responses or oxidative damage (all *P* > 0.123).

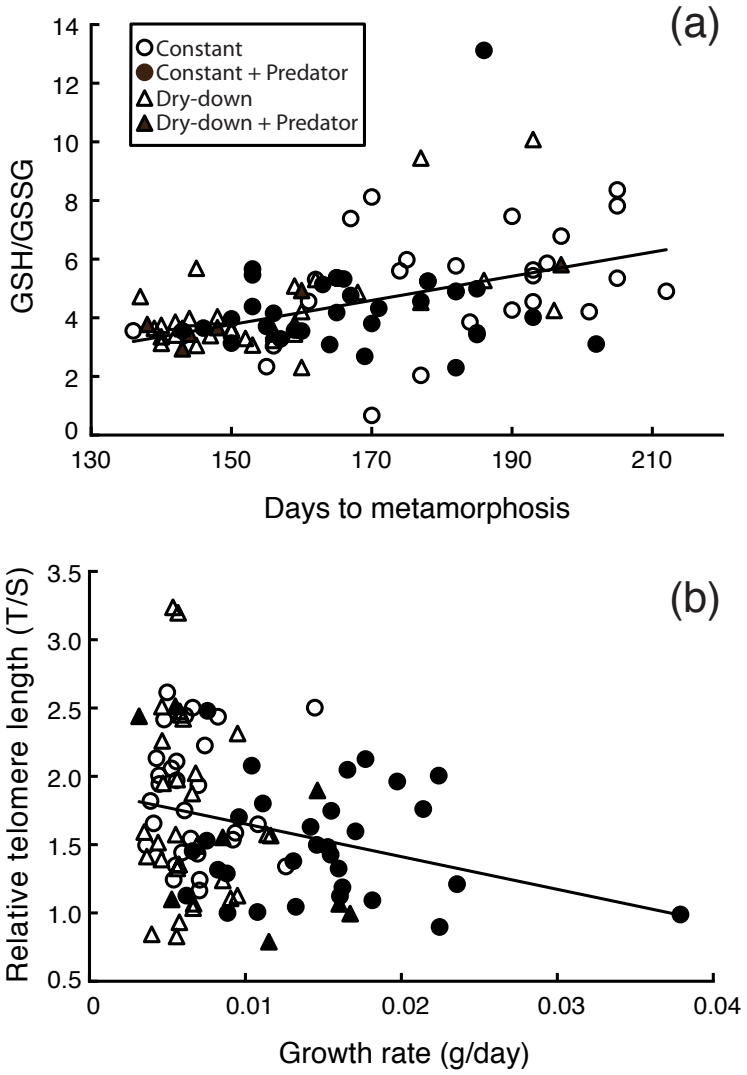

Figure 3. Regressions between (a) days to metamorphosis and GSH/GSSG ratio (R^2 = 0.19, P < 0.001) and between (a) growth and telomere length (R^2 = 0.07, P = 0.008). Regression lines show the correlation between physiological and life-history traits measured in *Pelobates cultripes* juveniles surviving all four experimental groups combined: constant water level, constant water level plus predator, dry-down, and dry-down plus predators. Cellular oxidative stress normally results in decreased GSH/GSSG ratio, whereas telomere shortening is a reliable indicator of individual/cellular senescence. The GSH/GSSG ratio increased with increased length of the larval period, whereas telomere length was shortened with increased growth rate.

Pond drying did not result in decreased relative telomere length (df = 1, 107; χ^2 = 0.422, P = 0.516; Figure 2d). However, individuals surviving predators had lower relative length (15.17 % on average; df = 1, 107; χ^2 = 6.892, P = 0.009; Figure 2d). We found no significant interaction in relative telomere length between both factors (df = 1, 107; χ^2 = 0.447, P = 0.504; Figure 2d).

We found a positive correlation between GSH/GSSG ratio and duration of the larval period (Figure 3a), i.e. tadpoles with faster developmental rates showed lower GSH/GSSG values (R^2 = 0.19, P < 0.001). This correlation was significant in constant water, low water, and low water plus predator treatments (R^2 = 0.18, 0.33, and 0.83 respectively, all P < 0.021) but not in the predator treatment (R^2 = 0.04, P = 0.320). GSH/GSSG ratio and growth rate did not correlate significantly (P = 0.156). On the other hand, relative telomere length correlated negatively with growth rate although the correlation had a low explanatory power (R^2 = 0.07; P = 0.008: Figure 3b). We observed a possible outlier in this relationship, with a high leverage on the analysis, showing an elevated growth rate (0.037 g/day). Such a growth rate, however, is within the normal range of growth rates observed for this species, and the individual metamorphosed with 6 g, which is also within the normal size range for metamorphosing individuals in this species. Therefore, we had no biological reasons to discard this data point. Nonetheless, we repeated the analyses without this data point and found that relative telomere length still showed a significant correlation with growth rate, even though the explanatory power was even lower (R^2 = 0.05; P = 0.024). Relative telomere length did not correlate with duration of the larval period.

Discussion

In our experimental setup, spadefoot toad larvae experienced environments very similar to realistic conditions found in natural ponds, and consequently the treatments comprising dry-down, predator presence, and especially the combination of both, considerably reduced larval survival. Such differential survival could have had a direct effect on the values observed for the physiological parameters recorded. However, the surviving individuals from the different treatments clearly differed in the level of oxidative stress experienced and in their relative telomere length, indicating that pond drying and reduced competition affected growth and development in rather different ways and with different physiological consequences.

Pond drying reduced larval survival and induced accelerated development at the expense of truncated growth and a smaller size and body mass at metamorphosis. These results are consistent with previous studies analysing plasticity in life history-traits in tadpoles exposed to pond drying (Ritcher-Boix et al. 2011; Gomez-Mestre et al. 2013). Smaller size at metamorphosis correlates negatively with fitness components in different taxa such as insects (Taylor et al. 1998), fish (Ryding and Skalski, 1999), and other amphibians (Gomez-Mestre and Tejedo, 2003). Developmental acceleration in response to pond drying also caused depletion of fat bodies. Fat bodies are the main lipid storage in amphibians and are determinant of juvenile viability, especially for species that estivate or hibernate after metamorphosis like *P. cultripes* (Reading, 2007). Moreover, developmental acceleration in response to pond drying induced higher activity of antioxidant enzymes, particularly GPx and GR. Such enhanced antioxidant activity is likely to have been caused by increased metabolic rate as a consequence of increased corticosterone levels,

and the consequent increase in ROS production (Gomez-Mestre et al. 2013). However, the redox imbalance seemed to be managed by the antioxidant enzymes as we found no signs of oxidative damage, as indicated by the lack of lipid peroxidation alteration. Moreover, juveniles did not seem to experience intense oxidative stress since GSHt, or GSSH levels remained unaltered. Despite the patent poorer condition of juveniles emerging from the experimental pond-drying regime in terms of size, body mass, fat storage, and oxidative stress, their relative telomere length did not vary. This lack of effect on relative telomere length despite incurring in oxidative stress may be due to successful antioxidant activity of enzymes that avoided oxidative damage in the cells.

Amphibian larvae respond to the non-lethal presence of predators by reducing their activity and modifying their morphology (Benard, 2004). Nevertheless, despite these inducible defences, free ranging predators may still have a strong thinning effect on tadpole density, as occurred in this study where the surviving larvae had higher availability of resources and lower intraspecific competition than those not exposed to predators. Consequently, they completed development under relaxed competition and reached metamorphosis quickly, attaining large sizes over the course of a short larval phase. They also metamorphosed with ample fat reserves. This enhanced growth rate was associated with lower antioxidant activity of GR and elevated GSH_t levels. GR activity is essential for removal of reactive oxygen species, as it reduces oxidized glutathione (GSSG), which causes oxidative damage in the cells. Previous studies have described decreased activity of antioxidant enzymes (Blokhina et al. 2003; Monaghan et al. 2009), as for example those from tadpoles exposed to herbicide, who decreased their CAT, SOD or GR activities (Burraco et al. 2013), or cold-stressed rats which decreased CAT and

GPx activities (Kaushik and Kaur, 2003). Reduced antioxidant activity compared to benign control conditions could be caused by enzyme inactivation due to excess lipid peroxides and ROS production (D'Autréaux and Toledano, 2007). In the same individuals, we also observed elevated GSH_t levels, one of the most important scavengers of ROS. The maintenance of GSH_t levels is crucial in preventing oxidative stress because GSH acts as a chain-breaker of free radical reactions and also because it is the substrate for GPx (Lesser, 2006). For example, increased GSH_t has been related to high rates of cellular proliferation in human hepatocellular carcinoma (Huang et al. 2001), and is postulated as a mechanism that modulates cell growth by interacting with ROS preventing damage to proteins or DNA and by participating in DNA repair (Huang et al. 2001). Observed increments in GSH_t levels associated with elevated growth and developmental rates in our experiment are thus congruent with a scenario of oxidative stress, as suggested by the inactivation of antioxidant enzymes, likely due to high ROS production. Tadpoles that survived predators grew quickly and accumulated large fat reserves. The accumulation of fats is linked to increased oxidative stress as well as to elevated GSH_t levels, which participates in the improvement of redox imbalance (Savini et al. 2013). Moreover, the rate of GSH_t synthesis depends on cysteine availability that is obtained directly from the food (Isaksson et al. 2011), which may explain elevated GSH_t levels in survivors against predators due to their higher resource availability. However, we only detected a non-significant increase of lipid peroxidation in individuals surviving predators. This may be due to the low specificity of the thiobarbituric acid reactive substances (TBARS) assay, as in this test TBA reacts with a variety of compounds other than malondialdehyde (MDA) like sugars or amino acids, likely interfering its measurement (Grotto et al. 2009).

Telomere sequences are rich in guanine content and highly sensitive to oxidative stress, which causes single-strand breaks that entail deficient repair of telomeric sequences (Houben et al. 2008; Monaghan et al. 2009). It has often been suggested that a trade-off between ROS production and lifespan exists, since oxidative stress is one of the main mechanisms explaining ageing at the cellular level (Dowling and Simmons, 2009; Salmon et al. 2011). In addition to unbalanced ROS, telomere shortening seems to be associated with increased growth rate (Hall et al. 2004; Monaghan et al. 2008; Lee et al. 2012). This is probably a consequence of biochemical and physiological alterations at the cellular level, as well as of increased cell replication during enhanced growth (Jennings et al. 2000; Lin et al. 2012).

Nevertheless, most analyses on oxidative stress and telomere shortening have been conducted on birds or mammals. Development and growth are normally tightly correlated in those groups and therefore it is difficult to disentangle whether oxidative stress and telomere shortening are primarily related to enhanced developmental rate, increased growth rate, or both. In contrast, amphibian larvae can largely decouple differentiation rate and growth rate depending on the environmental conditions (Gomez-Mestre et al. 2010). Thus, amphibian larvae can typically grow for extended periods of time without advancing in developmental stage, or accelerate development and trigger an early metamorphosis at the expense of truncating growth if conditions worsen. Here we found evidence for increased ROS production as a consequence of developmental acceleration in individuals exposed to pond drying, as indicated by increased activity of antioxidant enzymes. However, individuals undergoing developmental acceleration showed no sign of either oxidative damage or alterations in relative telomere length. In contrast, opposite effects

were found in response to predators, as enhanced growth rate in surviving juveniles against predators resulted in individuals experiencing increased GSH_t and decreased relative telomere length. These results indicate that the rate of cell replication during enhanced growth in early larval stages had a direct bearing on telomere shortening whereas increased metabolic activity as a consequence of just accelerated development did not, although it positively correlated with GSH/GSSG ratio.

In sum, pond drying reduced larval survival and induced accelerated development in spadefoot toad tadpoles, causing oxidative stress, reduced size at metamorphosis and diminished fat reserves, all of which is known to reduce short-term odds of survival to first reproduction in amphibians (Scott et al. 2007). Conversely, predation reduced greatly intraspecific competition resulting in fast growing tadpoles that metamorphosed large and with ample fat reserves. However, these individuals showed telomere shortening and showed also some signs of oxidative damage, which suggests that despite their greater odds of survival in the short- and mid-term, they may face reduced lifespan. As further studies on oxidative stress and relative telomere length in complex life cycles species accumulate, we will be able to confirm this pattern where short-term survival is determined by post-metamorphic traits whereas lifespan is linked to the physiological consequences of developmental responses to environmental conditions during early ontogenetic stages.

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Additional File 1

Oxidative stress assays

We determined total protein content though standard Bradford's method (Bradford, 1976). We quantified indirectly catalase (CAT) activity by measuring catalytic activity (Cohen and Somerson, 1969). According to this procedure, we used potassium permanganate ($KMnO_4$) as an oxidizing and colored agent, reacting with hydrogen peroxide (H_2O_2), the catalase substrate. The reduced $KMnO_4$ gives a red color that can be read at 480 nm five minutes after $KMnO_4$ is added. Standard curves were prepared using commercial catalase (SIGMA – 60634). Catalase activity is expressed as U/mg of total protein. Superoxide dismutase (SOD) activity was determined indirectly (Cord and Fridovich. 1969) by measuring the inhibition rate of cytochrome C reduction. Cytochrome C is oxidized by superoxide radicals (O_2^-) except in the presence of SOD which competes for O_2^- generated by xanthine and hypoxanthine action and reduces cytochrome C and produces hydrogen peroxide (H_2O_2) and oxygen. One unit of SOD is defined as the amount of enzyme that inhibits the rate of reduction of cytochrome C by 50 % at 25 ºC at 550 nm. The quantification of glutathione peroxidase (GPx) was according to the methods developed by Paglia and Valentine (1967). The oxidized glutathione (GSSG) is continually reduced due to an excess of glutathione reductase (GR) producing a constant level of reduced glutathione (GSH), which requires NADPH. We monitored NADPH oxidation spectrophotometrically at 340 nm. For GR determination we measured the decrease in absorbance at 340 nm due to NADPH oxidation (Cribb et al. 1989). We also measured lipid peroxidation according to the method developed by Buege and Aust (1978).

Malondialdehyde (MDA) is one product of lipid peroxidation which reacts with thiobarbituric acid, reporting a red product that absorbs at 535 nm. For the calculation of MDA concentration (in nmol MDA/mL) we measured the optical density values for the blank and for the calibration curve and estimated nmol MDA/mL by comparing with the calibration curve. For the determination of total glutathione levels we used the protocol developed by Galván et al. (2010). Homogenates were diluted (1:10, w/v) and homogenized in a stock buffer (0.01 M PBS and 0.02 M EDTA). We prepared three working solutions by using the same stock buffer as follows: (A) 0.03 mM of NADPH, (B) 6 mM 5,5'-Dithiobis(2-nitrobenzoic acid) (DNTB), and (C) 50 units of GR/mL. Solution A and B were mixed at a ratio of 7:1 respectively and 160 µL of this mixture was added to 40 µL of supernatant. After 15 seconds, we added 20 µL of the solution C and we read absorbance at 405 nm after 30 and 60 seconds. We determined the total concentration of glutathione by comparing the changes in absorbance between two readings, according to a standard curve generated by serial dilution of glutathione from 1 mM to 0.031 mM.

Additional File 2

Statistical results of measurements obtained in surviving spadefoot toad juveniles (*Pelobates cultripes*) exposed to pond drying and predators in a 2x2 experimental design in 500-L tanks. Number between parenthesis denote the number of animals used per treatment, according to the following order: high water no predator, low water no predator, high water no predator, and low water no predator. The variables measured were: survival, days to metamorphosis, growth rate, snout-to-vent (SNV) length, body mass, and fat storage. Moreover, we measured the following physiological parameters: catalase (CAT) activity, glutathione peroxidase (GPx) activity, glutathione reductase (GR) activity, superoxide dismutase (SOD) activity, malondialdehyde (MDA) concentration, reduced glutathione (GSH) concentration, the ratio of reduced to oxidized glutathione (GSH/GSSG), and the relative telomere length.

Life-history traits and fat storage

Survival	**df (240,240,240,240)**	**χ^2**	***P*-value**
pond drying	1, 959	12.005	< 0.001
predator	1, 959	30.331	< 0.001
pond drying * predator	1, 959	0.241	0.623
tank (random factor)	1, 23	11.726	< 0.001
family (random factor)	1, 9	8.895	0.003
Time to metamorphosis	**df (138,108,58,20)**	**χ^2**	***P*-value**
pond drying	1, 323	15.265	< 0.001
predator	1, 323	17.713	< 0.001
pond drying * predator	1, 323	0.99	0.32

tank (random factor)	1, 23	7.043	0.008
family (random factor)	1, 9	3.101	0.078
Body mass	**df (124,89,49,18)**	**χ^2**	***P*-value**
pond drying	1, 279	10.653	0.001
predator	1, 279	24.451	< 0.001
pond drying * predator	1, 279	0.0798	0.778
tank (random factor)	1, 23	19.294	< 0.001
family (random factor)	1, 9	12.581	< 0.001
SNV length	**df (124,89,49,18)**	**χ^2**	***P*-value**
pond drying	1, 279	6.539	0.011
predator	1, 279	23.755	< 0.001
pond drying * predator	1, 279	1.102	0.294
tank (random factor)	1, 23	23.041	< 0.001
family (random factor)	1, 9	11.178	< 0.001
Growth rate	**df (124,89,49,18)**	**χ^2**	***P*-value**
pond drying	1, 279	6.009	0.014
predator	1, 279	28.586	< 0.001
pond drying * predator	1, 279	2146.5	< 0.001
tank (random factor)	1, 23	21.344	< 0.001
family (random factor)	1, 9	6.8131	0.009
Fat storage	**df (33,34,31,11)**	**χ^2**	***P*-value**
pond drying	1, 108	6.166	0.013
predator	1, 108	3.886	0.049
pond drying * predator	1, 108	1.944	0.163
tank (random factor)	1, 23	9.265	0.002
family (random factor)	1, 9	0	0.999

CAT	**df (32,32,32,9)**	**χ^2**	***P*-value**
pond drying	1, 104	0.302	0.582
predator	1, 104	1.707	0.191
pond drying * predator	1, 104	1.9693	0.16
tank	1, 23	5.63	0.018
family	1, 9	0.477	0.49
GPx	**df (29,31,31,9)**	**χ^2**	***P*-value**
pond drying	1, 99	9.54	0.002
predator	1, 99	0.461	0.497
pond drying * predator	1, 99	2.385	0.123
tank	1, 23	0.039	0.843
family	1, 9	0	1
GR	**df (32,32,32,9)**	**χ^2**	***P*-value**
pond drying	1, 104	6.647	0.01
predator	1, 104	7.63	0.006
pond drying * predator	1, 104	0.683	0.4085
tank	1, 23	0	1
family	1, 9	0.176	0.674
SOD	**df (32,32,32,9)**	**χ^2**	***P*-value**
pond drying	1, 104	2.297	0.13
predator	1, 104	0.907	0.341
pond drying * predator	1, 104	0.186	0.667
tank	1, 23	1.843	0.175
family	1, 9	4.341	0.037
MDA	**df (32,32,31,9)**	**χ^2**	***P*-value**
pond drying	1, 103	0.084	0.772
predator	1, 103	3.504	0.061
pond drying * predator	1, 103	1.06	0.3031
tank	1, 23	4.366	0.037

family	1, 9	10.756	0.001
GSHt	**df (29,32,31,8)**	**χ^2**	***P*-value**
pond drying	1, 99	2.032	0.154
predator	1, 99	4.468	0.034
pond drying * predator	1, 99	1.695	0.195
tank	1, 23	0	1
family	1, 9	2.87	0.09
GSH/GSSG ratio	**df (29,32,31,8)**	**χ^2**	***P*-value**
pond drying	1, 99	3.499	0.061
predator	1, 99	2.168	0.14
pond drying * predator	1, 99	0.17	0.68
tank	1, 23	0	1
family	1, 9	0	1
Relative telomere length	**df (34,33,32,11)**	**χ^2**	***P*-value**
pond drying	1, 107	0.422	0.516
predator	1, 107	6.892	0.009
pond drying * predator	1, 107	0.447	0.504
tank	1, 23	0	1
family	1, 9	0	1

CHAPTER 3

Physiological maintenance costs of plasticity in amphibian larvae against two common environmental risks

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Abstract

Plasticity benefits processes such as diversification and speciation by favoring a better phenotype-environment matching under changing environments. However, genotypes of a given species usually differ in their degree of phenotypic plasticity. Costs of maintaining the sensory and response machinery of plastic traits have been proposed as a plausible explanation by evolutionary models to understand differences on plasticity within populations, although only a few studies have detected it empirically. Surprisingly no studies have attempted to quantify maintenance costs of plasticity from a physiological or biochemical point of view. Here we test for physiological maintenance costs of developmental, growth, and morphological plasticity in amphibian larvae against two main factors that compromise their survival: desiccation and predators. Particularly, we determined physiological parameters related to individual fitness such as weight at metamorphosis, fat reserves, standard metabolic rate, antioxidant responses, and immune status. We found through model selection attending to Akaike information criterion that plastic responses against both factors resulted costly in terms of oxidative stress, growth, or immune unbalances. Moreover, we detected trade-offs of developmental plasticity when larvae responded to each factor, which could suggest constrains on the evolution of adaptive

plasticity. Therefore, our results indicate that plasticity can be physiologically costly which might help to understand plasticity evolution and its limits.

Keywords: Amphibians; Evolutionary physiology; Phenotypic plasticity; Plasticity costs; Pond drying; Predators

Introduction

Phenotypic plasticity can favor adaptation and diversification by granting a better match between individual phenotypes and local environments when environments are heterogeneous. Nevertheless, the evolutionary benefits of plasticity might be reduced by costs of possessing the ability to develop alternative phenotypes, known as 'maintenance costs' (DeWitt, 1998; Auld et al. 2010; Ledón-Rettig et al. 2010). Such costs are associated solely to the maintenance of the mechanisms needed to detect and respond to environmental inputs, and are different from 'production costs', which are costs paid by organisms during the actual production or expression of alternative phenotypes (DeWitt, 1998). For instance, many plants maintain the capacity for detecting variation in the red:far red light spectrum using phytochromes, and to respond by elongating stems hence avoiding the shade casted by neighboring competitors (Smith, 2000). However, we can distinguish between the costs of maintaining the capacity for detecting and activating the plastic response (maintenance costs, paid constantly) and the costs incurred in the actual production of the elongated stems (production costs, only paid when the appropriate environmental cue is detected).

Models show that costs of maintaining the sensory machinery and the mechanisms to produce a plastic response would hinder the evolution of plasticity so that plasticity would only be favored when its

benefits outweigh its costs, as in highly heterogeneous environments (Scheiner and Berrigan, 1998; Gomez-Mestre and Jovani, 2013; Chevin and Lande, 2015). High maintenance costs would therefore constrain the evolution of plasticity (Relyea, 2002; Murren et al. 2015), just as low genetic variation would (Schlichting and Pigliucci, 1998). Consequently, high plasticity costs often result in trait canalization and loss of plasticity (Chevin et al. 2010; Gomez-Mestre and Jovani, 2013; Hollander et al. 2015). In addition, natural selection is expected to reduce maintenance costs by offsetting costly mechanisms during ontogeny, through epigenetic regulation of gene expression or via genetic network rearrangement (Hittinger and Carroll, 2007; Van Burskirk and Steiner, 2009; Murren et al. 2015).

A number of studies in the last two decades have tried to quantify maintenance costs of plasticity, although only a few of them have succeeded at empirically detecting them (e.g. DeWitt, 1998; Relyea, 2002; Steinger et al. 2003). Van Buskirk and Steiner (2009) meta-analyzed 27 studies that reported 536 separate traits for which maintenance plasticity costs were examined, both in plants and animals. They concluded that fitness costs of plasticity were relatively mild (28.6% of the total negative fitness selection coefficients), and costs only seemed to be larger under stressful environmental conditions. Hence, costs of plasticity often appear to be of little importance or at least hard to detect (Van Buskirk and Steiner, 2009; Murren et al. 2015). Nevertheless, a few studies described that species with high investment in structures key such as large brains or complex immune responses experience considerable maintenance costs of plasticity associated with such structures (Laughlin et al. 1998; Snell-Rood, 2012). A possible explanation for the apparent lack of such costs in plastic traits is that selection would purge genotypes for which plasticity costs were elevated, for example by removing interactions between plasticity loci and loci affecting fitness

(DeWitt et al. 1998; Murren et al. 2015). Nevertheless, in those cases plasticity costs should recurrently re-emerge after recombination (Van Kleunen and Fischer, 2007). Moreover, costs can be uniformly distributed within populations, making it difficult to assess maintenance costs through comparison across genotypes within populations (Van Buskirk and Steiner, 2009). Furthermore, it has been argued that plasticity may only become costly under ecological scenarios that are not easily replicated experimentally (Agrawal, 2001).

Auld et al.(2010) suggested that "for maintenance costs, plastic individuals must invest resources in maintaining the molecular physiological 'machinery' needed to detect, monitor and respond to environmental conditions", however, the physiological basis of these costs, and whether they are analogous or different in nature to physiological costs of production of plastic responses, remains largely unexplored. Maintenance costs of plasticity have usually been sought for through direct or indirect measurements of fitness such as growth, development, survival, productivity, or fecundity rate (Scheiner and Berrigan, 1998; Dorn et al. 2000; Relyea, 2002; Steinger et al. 2003). In a few instances, maintenance costs were evident when it was found that more plastic genotypes within populations resulted in reduced growth or lower survival (DeWitt, 1998b; Relyea 2002; Steinger et al. 2003). However, if selection erodes maintenance costs of plasticity, such broad fitness-related phenotypic consequences of plasticity may become quickly buffered, although perhaps more subtle costs persist. The arguments for the existence of maintenance costs are often framed in terms of energy allocation or physiological tolls for maintaining sensory machinery and the mechanisms for phenotypic alterations, and yet, to the best of our knowledge, there are no studies that address maintenance costs from a physiological point of view. In many instances the physiological regulation and consequences of adaptive plasticity are well known (e.g.

Shmitt et al. 1999; Stoks et al. 2006; Sommer and Ogawa, 2011), facilitating the formulation of specific hypotheses regarding the possible nature of associated costs.

In animals, plastic responses are commonly regulated by neuroendocrine pathways whose activation implies production costs in terms of altered life-history traits, metabolism, or fitness (Johansson, 2002; Benard, 2004; Gervasi and Foufopoulos, 2007). Maintenance of the machinery required to both detect and respond to environmental inputs may thus happen at the expense of metabolic or immune costs. Maintenance of such machinery might require increased metabolic rate (Wingfield and Romero 2001) and up-regulation of processes like gluconeogenesis and fat catabolism that lead to overproduction of reactive oxygen species (ROS), unbalancing the cellular redox status and damaging cell structures (Monaghan et al. 2009; Costantini et al. 2010). Moreover, maintenance of energetically expensive processes may also divert resources from other important functions such as the immune system (Blas et al. 2006), potentially generating a trade-off between plasticity and immunocompetence.

Here we tested the existence of physiological costs of developmental plasticity in response to two common risks to larval amphibians in temporary ponds: predator presence and pond drying (Skelly, 1997). Responses against these factors have the particularity to occur in opposite directions in terms of development, growth, and induced morphology (Denver et al. 1998; Relyea, 2007; Richter-Boix et al. 2011). We hypothesized that, if plasticity is costly, high plastic sibships will pay a physiological toll in terms of increased metabolism and compromised immune status. Moreover, since pond drying and predators alter larval life-history traits in opposite directions, we would expect a certain trade-off between the responses to each of these factors so that highly responsive genotypes against one factor would show lower plastic ca-

pacity to respond to the other factor. To test these hypotheses, we collected clutches of western spadefoot toads (*Pelobates cultripes*) from three different locations and assessed their degree of developmental plasticity against each factor by raising the larvae to metamorphosis under either high water or dry-down conditions, and presence or absence of predator chemical cues. Simultaneously to that experiment aimed at describing the reaction norms for each sibship, we ran a second experiment where we examined among-sibship variation in physiological parameters under benign conditions of high water level and absence of predators. We determined physiological parameters related to individual fitness such as weight at metamorphosis, fat reserves, standard metabolic rate, antioxidant responses, and immune status.

Material and methods

In March 2013 we collected portions of a total of 20 sibships of *Pelobates cultripes* from three different localities: 6 sibships from Doñana National Park (Huelva), 9 from Doñana Biological Reserve (Huelva), and 5 from Sierra Norte Natural Park (Sevilla), all in southwestern Spain. Our aim in including clutches from different localities in the experiment was not to test hypotheses regarding geographic variation, but to ensure there was sufficient among-sibship variation to test for plasticity costs.

Pelobates cultripes have a long larval period (4-6 months; Gomez-Mestre and Buchholz, 2006; Zeng et al. 2014), and are commonly exposed to both risk of predation and risk of pond drying. We collected egg clutches from temporary ponds that usually contain native invertebrate predators. Sibships were individually maintained until hatching in 10 L plastic buckets filled with 5 L of carbon-filtered dechlorinated tap water in a walk-in climatic chamber set at 18 ºC. Once hatched,

90 tadpoles of each sibships were haphazardly separated into groups of 3 tadpoles and assigned to different treatments to quantify reaction norms of plasticity against each factor (see below). Similarly, 10 tadpoles from each sibship were individualized for quantification of plasticity maintenance costs (see below). Tadpoles were put in 3 L buckets (168 mm diameter x 184 mm high) filled with 2.7 L of dechlorinated tap water in climatic chambers set at 24 ºC with a 12:12 light:dark photoperiod and were fed rabbit chow *ad libitum*. We also collected *Dytiscus circumflexus* larvae as natural predators of *P. cultripes* tadpoles within all locations used in the experiment. *Dytiscus* larvae are voracious predators of amphibian larvae. They have the ability to inject digestive enzymes to their prey through their mandibles, and a single *Dytiscus* larva is able to consume 300-900 tadpoles 10-20 mm in length in the course of their development (Inoda et al. 2009).

Reaction norm determination

To assess the level of plasticity in morphology, developmental rate, and growth rate, we experimentally exposed tadpoles from each sibship to chemical cues from predators or to simulated pond drying. The experimental design included consisted of three treatments: high water level (2.7 L) without predator cues ('control' treatment); low water (300 mL) without predator cues ('pond drying'); and high water level plus predator cues ('predator presence'). We set 10 replicates per sibship (three individuals/bucket) and treatment combination for a total of 600 experimental units, randomly arrayed throughout three climatic chambers set at 24 ºC. The experiment started when tadpoles reached 25 Gosner stage (free feeding and active swimming; Gosner, 1960). Water was renewed once a week, and water levels were readjusted for each treatment as appropriate. All tadpoles were fed rabbit chow *ad libitum*.

In the control treatment water level was kept constant readjusting to 2.7 L (16 cm high water column). Tadpoles in the pond drying treatment were raised in a high water volume until at least one tadpole in the bucket had reached 35 Gosner stage (all five digits in the hind limb visible), and then we dropped the water level to 300 mL, representing a 3 cm high water column. This stage was chosen because *Pelobates cultripes* larvae at that stage show the highest capacity for developmental acceleration (Kulkarni et al. 2011). In the predator treatment we added water borne predator cues mixed with alarm cues from conspecific tadpoles twice a week. To obtain predator kairomones we maintained 12 *Dystiscus* larvae individually in 1 L buckets filled with 0.8 L dechlorinated water. We fed each *Dytiscus* larvae one *P. cultripes* tadpole every other day. Twice a week, we filtered and pooled the water from each individual predator container to obtain a homogeneous mix and avoid biases due to predator identity. All experimental containers in the 'predator' treatment received 40 mL of this pool of water containing predator kairomones and alarm cues, whereas the 'control' and 'pond drying' containers received 40 mL of clean water.

Two weeks after we initiated the 'pond drying' treatment, we randomly chose one tadpole per bucket to photograph laterally for morphometric analysis (see below). When the first tadpole of each bucket reached metamorphosis, i.e. 42 Gosner stage (forelimbs emerged and full tail remains), we put it in a 1 L bucket filled with 50 mL of water until it completed metamorphosis (46 Gosner stage, tail completely reabsorbed). Metamorphs at 46 Gosner stage were weighed to the nearest 0.0001 g using a high precision balance (CP324S, Sartorius). When the first individual of each bucket metamorphosed, we discarded the other two individuals and we used them for other experiment. If any of the three individuals of each bucket died before one reached metamorphosis we discarded this replicate for further analysis.

Morphometric analysis

We applied geometric morphometrics methods to describe shape variations in tadpoles across treatments. We photographed the left side of each tadpole and all photos were scaled using a grid of know dimensions. We delimited the shape of tadpoles by digitizing 8 landmarks and 15 sliding semilandmarks (see Figure in Additional File 1) with tpsDig2 software (Rohlf, 2010; Rohlf. 2010b). Landmarks were chosen considering their ability to capture the overall body shape of tadpoles while satisfying statistical restrictions associated with geometric morphometrics (Rufino et al. 2006). In order to control for postural changes in shape unrelated to treatment we corrected landmark position with a quadratic function using the *unbend* option in tpsUtil (Rohlf, 2010). We performed generalized procrustes analysis (Rohlf and Slice, 1990) using the package *geomorph*, version 3.0 (Adams et al. 2016) in R. Because there was an allometric component of shape variation (test for the association between centroid size –CS– and shape: $P = 0.001$), we calculated residuals from a linear regression of shape on log(CS). We then used principal components analysis (i.e. relative warps, abbreviated as RWs) to determine "allometry-free" body shape variation among specimens. We included in our analyses the first four relative warps, which explained the 30.5%, 20.8%, 11.5%, and 9.6% of the total morphometric variance, respectively. These warps explained variation in common morphological features previously described for amphibian larvae (Hossie et al. 2010; Orizaola et al. 2013).

Determination of physiological maintenance costs of plasticity

Two months after tadpoles hatched we measured several physiological parameters closely associated with body condition and indi-

rectly with individual fitness. In particular we determined standard metabolic rate, fat reserves, the activity of four antioxidant enzymes (catalase, superoxide dismutase, glutathione dismutase, glutathione peroxidase), malondialdehyde levels (a product formed during lipid peroxidation), reduced/oxidized glutathione concentrations, and leukocyte count, in addition to body mass. For each tadpole, we first measured its standard metabolic rate (see below), then blotted dry the excess water and weighed it. Each tadpole was then euthanized with MS-222 (SIGMA) immediately prior to blood extraction to estimate leukocyte proportion, and to fat bodies dissection. Fat bodies were also weighed to the nearest 0.0001 g. Finally, each tadpole was eviscerated and then snap frozen and stored at -80 ºC until assayed for oxidative stress.

Standard metabolic rate

We determined metabolic rate using an aquatic respirometer consisting on a set of ten optical sensors (Oxy 10-PreSens), which register simultaneously the oxygen concentration (mg/L) at the entrance and the exit of five plexiglass chambers (44 mm in diameter x 163 mm long cylinders). The ten optical sensors were connected to an oxygen meter (Oxy 10-PreSens, Germany) and oxygen level was recorded every 15 seconds. These sensors are optodes that do no consume oxygen during measurements and present a long-term stability. We calibrated the respirometer once daily using sodium sulphite and oxygen saturated water to achieve 0 and 100 % oxygen concentrations. All calibrations and measurements were performed at 24 ºC. Once calibrated, tadpoles were individually introduced in the chambers and the oxygen consumption was recorded for 25 minutes. We discarded the first 5 minutes of data recording, considered

as acclimation period (Burraco et al. 2013; Burraco and Gomez-Mestre, 2016), and we calculated metabolic rate following Alvarez et al. (2006):

$VO_2 = V_w \times \Delta C_w$

where VO_2 (μg h^{-1}) is the SMR measured as rate of oxygen consumption, Vw is the flow rate through the chamber (1 h^{-1}), and ΔCw is the instantaneous difference in O_2 concentration between inflow and outflow.

Leukocyte count

The immune state of individuals can be altered by shifting the proportion of white blood cells, as described in hematological assessments of stress (Davis et al. 2008). We therefore estimated relative abundance of leukocytes (i.e. granulocytes, lymphocytes and monocytes) and also erythrocytes through flow cytometry (Uchiyama et al. 2005; Burraco et al. 2017) as a proxy for immune state. Blood was obtained via cardiac puncture with a non-heparinized syringe (BD Micro-Fine insulin 29G 0.5 mL) and introduced in a heparinized tube kept on a layer of tissue paper over ice to prevent direct contact with the ice and avoid hemolysis. We diluted 1 μL of blood in 1 mL of Hanks' balanced salt solution and mixed it with 1 μL of 3,3'-Dipentyloxacarbocyanine iodide ($DiOC_5(3)$, Sigma-Aldrich, St. Louis, MO) previously diluted 1:10 in absolute methanol. $DiOC_5(3)$ is a fluorescent lipophilic dye and is highly fluorescent and photostable when incorporated onto biological membranes. The mixture of blood, Hanks' solution and $DiOC_5(3)$ was vortexed to ensure a good mix. After 2 min of adding the $DiOC_5(3)$ we determined leukocyte profiles passing the mix through a flow cytometer (Guava Easy Cyte Plus, Guava Technologies, Hayward, California, USA). We counted a maximum of 25,000 cells per sample. We differentiated four cell types according to gating strategy described in Burraco et al. (2017): erythrocytes, lymphocytes, granulocytes, and monocytes. Because of the

low number of monocytes in amphibian blood (Davis et al. 2008) we discarded them for further analysis. We used the granulocyte-to-lymphocyte ratio as well as the absolute count of granulocytes and lymphocytes (cells per microliter of blood) to assess the immune state of individuals (Davis et al. 2008).

Fat reserves

The major triglyceride storage in amphibian larvae is located in the abdominal area in the form of fat bodies, which are essential for a successful metamorphic transition to the terrestrial phase (Scott et al. 2007). Tadpoles were thawed and dissected for fat storages quantifications. Fat storages were weighed in an electronic balance to the nearest 0.0001 g (CP324S, Sartorius).

Oxidative stress

We quantified the activity of catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPx), and also malondialdehyde (MDA) and total reduced and oxidized glutathione (GSH_t and GSSG, respectively) concentration. Eviscerated individuals were dissected and homogenized in a buffered solution (1:4; homogenates:solution) to inhibit proteolysis (100 mM Tris-HCl with 0.1 mM EDTA, 0.1% triton X-100, pH 7.8 and 0.1 mM PMSF) using a Miccra homogenizer (Miccra D-1). Homogenates were centrifuged at 20,817 g for 30 min at 4 ºC and supernatants were aliquoted into 0.6 mL tubes and stored at -80 ºC. Total protein content was calculated using standard Bradford's protocol (Bradford, 1976).

We determined the activity of antioxidant enzymes as briefly described below. CAT activity was determined indirectly using a method

developed by Cohen and Somerson (1969). Potassium permanganate ($KMnO_4$) is an oxidizing agent that acts on H_2O_2 (reducing agent) producing H_2O_2 and O_2. This reduces $KmnO_4$ and produces a red product which absorbs at 480 nm. We performed standard curves by using commercial catalase (SIGMA-60634) by determining the absorbance at 480 nm five minutes after adding $KmnO_4$. We expressed the CAT activity as U / mg of total proteins. The activity of SOD was quantified following the protocol developed by Cord and Fridovich (1969). SOD inhibits the reduction of ferrocythocrome C by superoxide free radicals (O_2^-) because SOD produces hydrogen peroxide and molecular oxygen in the presence of O_2^-. One unit of SOD is defined as the amount of enzyme that inhibits the rate of reduction of ferrocythocrome C by 50% at 25ºC at 550 nm. We quantified indirectly GPx activity as described Paglia and Valentine (1967), by measuring NADPH oxidation at wavelength of 340 nm. NADPH reduces the product formed by GPx during the conversion of hydrogen peroxide into water (i.e. oxidized glutathione). On the other hand, GR produces reduced glutathione by oxidizing NADPH. We quantified GR activity by reading absorbance at 340 nm according to Cribb et al. (1989). We quantified the concentration of MDA as consequence of lipid peroxidation according to Buege and Aust (1978). MDA is a secondary product of lipid peroxidation, which reacts with thiobarbituric acid producing a red product that absorbs at 535 nm. We measured the optical density values for the blank and for one calibration curve and then MDA concentration (in nmol/ml) by subtracting the blank values to the absorbance of each sample, and comparing with the calibration values. Finally, we determined the levels of GSH_t and GSSG by using the protocol developed by Galván et al. (2010).

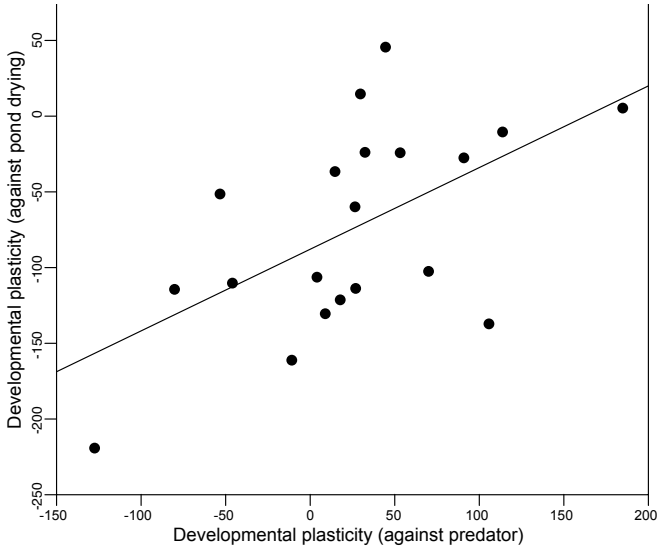

Figure 1. Developmental plasticity (in days) of 20 *Pelobates cultripes* sibships in response to reduced water level simulating pond drying (y-axis) and predator cues (x-axis). Negative values denote a developmental acceleration compared to control conditions (i.e. high water level and lack of predator cues).

Statistical analyses

All statistical tests were conducted in R (R Development Core Team 2014, version 3.3.1). Parametric assumptions were tested using Kolgomorov-Smirnov test for normality and Breusch-Pagan test for homoscedasticity. We used linear mixed models ('*lme4*' package) to test for differences in the degree of plasticity among sibships in response to each environmental factor testing for a 'treatment by sibship' interaction, and also by comparing AIC-differences between models. Sibship was always included as a random factor. We tested for significant differences in physiological measurements among sibships using

likelihood ratio tests in order to find candidate variables for inherent costs of plasticity. Body weight was taken into account as a covariate in statistical analyses of metabolic rate. Concentrations of MDA were log-transformed to fit parametric assumptions. Plasticity costs were evaluated with models that tested for associations between degree of plasticity across sibships and physiological descriptors of body condition or metabolic costs. We conducted model selection attending to Akaike information criterion (AIC; Akaike, 1973) with a correction for finite sample sizes (AICc) and following recommendations from Burnham and Anderson (2002) and also from Grueber et al. (2011). We only included plastic responses in the process of model selection for which we detected differences among sibships. Similarly, we only included physiological parameters that differed significantly among sibships (see Results section). We excluded GR values in the model selection since its values were collinear with SOD (GR-SOD correlation: R^2 = 0.11, *P*-value = < 0.0001) and GPx values (GR-GPx correlation: R^2 = 0.17, *P*-value = < 0.0001). All variables included in the global model were standardized with the function *scale* to allow better comparison of the estimates. We generated a global model for each type of plastic response observed (developmental, growth, and morphological) against the two environmental factors (pond drying and predators):

global.model: lm (plasticity ~ weight + SOD + CAT + GPx + MDA + G:L ratio, data = data)

We then used the function *dredge* implemented in the *MuMIn* package (version 1.15.6) to generate a submodel set from the global model. It resulted in 64 models that were restricted to the top 2-AIC_c models using the function *get.models* implemented in the same package. We then calculated the model average of the top 2AIC_c models with the function *model.avg*. Finally, the summary of the top models provided the estimates and the relative importance of each variable. For each

selected model, we determined R^2 and Δ-values (AIC-differences) compared to the global and null models.

When we found significant 'sibship by treatment' interactions in response to both environmental factors for any of the plastic traits studied, we also checked for trade-offs between the plastic responses to each factor by calculating the coefficient of correlation and its statistical significance (e.g. if high developmental plasticity in response to pond drying was or not associated with high developmental plasticity in response to predator cues).

Results

Plastic responses

Survival among tadpoles in the control treatment (83%) and the predator cues treatment (85.5%) were relatively high. However, a logistical error consisting in an excess of food supplied over a weekend resulted in an episode of mortality in the pond drying treatment that reduced survival to 43%. Food overabundance was a more severe problem for tadpoles in a smaller volume of water, and consequently that treatment was differentially affected. That episode of mortality reduced our statistical power to detect plastic changes in development, growth or morphology in response to pond drying, but in spite of it we still detected marked differences in plasticity among sibships both against pond drying and predator cues in terms of developmental, growth, and morphological responses (Table 1; see Additional File 2). Sibships differed in developmental plasticity, i.e. the timing of metamorphosis, both in response to pond drying and to predator cues, as indicated by significant 'treatment by sibship' interactions (df = 3; $\chi^2 = 76.75$; $P < 0.0001$ and df = 3; $\chi^2 = 114.75$; $P < 0.0001$, respectively; Table 1). Similarly, we found differences among

sibships in how their growth was altered in response to both pond drying and predator cues (df = 3; χ^2 = 16.23; P = 0.0012 and df = 3; χ^2 = 35.49; P < 0.0001, respectively; Table 1). In terms of morphological plasticity, we found no morphological alterations using the first relative warp (RW1) in response to neither pond drying (df = 3; χ^2 = 0.31; P = 0.9575; Table 1) nor predator cues (df = 3; χ^2 = 5.72; P = 0.1264; Table 1). Morphological changes represented by RW2 varied among sibships in response to predator cues (df = 3; χ^2 = 5.7146; P < 0.0001; Table 1), but did not vary among sibships facing pond drying (df = 3; χ^2 = 0.90; P = 0.826; Table 1). Similarly, RW3 showed a 'sibship by predator cues' interaction (df = 3; χ^2 = 13.21; P = 0.004; Table 1) but not a 'sibship by pond drying' interaction (df = 3; χ^2 = 4.08 ; P = 0.25; Table 1). The fourth relative warp (RW4) significantly varied among sibships both against pond drying and in the presence of predator cues (df = 3; χ^2 = 25.537; P < 0.0001 and df = 3 χ^2 = 15.151; P = 0.0017, respectively; Table 1).

Table 1. Pond drying and predator cues effects on development, growth rate, and morphology (first four Relative Warps, abbreviated RW) in *Pelobates cultripes* newly metamorphosed individuals (46 Gosner stage). A significant treatment-by-sibship interaction indicates differences among sibships in the degree of plasticity for a particular trait.

Trait	Effect	Df	Chi-sq	*P*-value	N
Development	pond drying * sibship	3	76.753	< 0.0001	244
	pond drying	1	51.869	< 0.0001	244
	sibship	1	50.396	< 0.0001	244
	predator * sibship	3	114.75	< 0.0001	328
	predator	1	5.7551	0.0164	328
	sibship	1	95.976	< 0.0001	328
Growth rate	pond drying * sibship	3	16.226	0.0011	231
	pond drying	1	29.143	< 0.0001	231
	sibship	1	7.3431	0.0067	231

	predator * sibship	3	35.49	< 0.0001	307
	predator	1	5.8291	0.0158	307
	sibship	1	35.02	< 0.0001	307
Morphology (RW1)	pond drying * sibship	3	0.3133	0.9575	311
	pond drying	1	11.126	0.0009	311
	sibship	1	0.2533	0.6148	311
	predator * sibship	3	5.7146	0.1264	311
	predator	1	2.4019	0.1212	311
	sibship	1	6.1833	0.0129	311
Morphology (RW2)	pond drying * sibship	3	0.8994	0.8256	311
	pond drying	1	2.8782	0.0898	311
	sibship	1	6.7779	0.0092	311
	predator * sibship	3	29.311	0.0092	311
	predator	1	10.083	0.0015	311
	sibship	1	28.29	< 0.0001	311
Morphology (RW3)	pond drying * sibship	3	4.0795	0.253	311
	pond drying	1	5.333	0.0209	311
	sibship	1	11.05	0.0009	311
	predator * sibship	3	13.21	0.0042	311
	predator	1	13.256	0.00027	311
	sibship	1	18.006	< 0.0001	311
Morphology (RW4)					
	pond drying * sibship	3	25.537	< 0.0001	311
	pond drying	1	2.5282	0.1118	311
	sibship	1	24.39	< 0.0001	311
	predator * sibship	3	15.151	0.0017	311
	predator	1	0.0965	0.7560	311
	sibship	1	13.435	0.0002	311

Physiological maintenance costs of plasticity

Tadpole survival was 98% during the two months that we raised them until we euthanized them to determine the physiological parameters of each sibship.

Table 2. Sibship effects on levels of fat reserves, body mass, metabolic rate, catalase activity (CAT), glutathione peroxidase activity (GPx), glutathione reductase activity (GR), superoxide dismutase activity (SOD), malondialdehyde (MDA), reduced glutathione (GSH), reduced:oxidized glutathione ratio (GSH:GSSG ratio), granulocyte-to-lymphocyte (G:L) ratio, and absolute lymphocyte and granulocyte count.

Physiological variable	Df	Chi-sq	*P*-value	N
Fat reserves	19	29.329	0.06098	167
Body mass	19	67.91	< 0.0001	195
Metabolic rate	19	16.539	0.621	179
CAT	19	53.92	< 0.0001	180
GPx	19	85.503	< 0.0001	183
GR	19	61.057	< 0.0001	184
SOD	19	75.588	< 0.0001	178
MDA	19	47.504	0.0003	184
GSH	19	21.643	0.3024	161
GSH:GSSG ratio	19	27.738	0.0886	155
G:L ratio	19	232.41	< 0.0001	196
Absolute lymphocyte count	19	15.602	0.6836	196
Absolute granulocyte count	19	8.0683	0.9860	196

We did not find significant differences among sibships in metabolic rate, fat reserves, GSH_t, GSG:GSSG ratio, or in the absolute count of lymphocytes or granulocytes (all $P > 0.0610$, Table 2). However, we found differences among sibships in body mass, activity of several

antioxidant enzymes (CAT, GR, GPx, SOD), in MDA, and in granulocyte-to-lymphocyte ratio (all $P \leq 0.0003$, Table 2). Model selection resulted in models indicative of associations between physiological parameters and the degree of plasticity across sibships, although such associations varied depending on the plastic trait and the environmental factor eliciting the plastic response (Table 3). We did not detect maintenance costs of developmental and growth plasticity in response to pond drying, indicated by the low explanatory power and low delta values of the selected models (Table 3). In contrast, developmental and growth plasticity in response to predators seemed to have associated physiological consequences. Thus, developmental plasticity in response to predator cues was associated with increased GPx activity, indicated by both delta-values and the R^2 values (Table 3). Plasticity in growth in response to predators were associated with changes in the granulocyte-to-lymphocyte ratio (Table 3). Also, morphological plasticity represented in relative warps RW3 and RW4 in response to predator cues had associated physiological consequences. Thus, plastic shifts in shape along RW3 was associated with increased GPx activity with an R^2 of 0.34, whereas RW4 was associated with MDA levels, GPx activity and SOD activity with an R^2 of 0.41 (Table 3).

Moreover, morphological plastic changes in response to pond drying (in RW4) were also associated with physiological alterations, like increased MDA level and SOD activity, but also to GPx and CAT activity and to a lesser degree to granulocyte-to-lymphocyte ratio and body mass (Table 3).

Table 3. Estimates, standard errors (SE), and relative importance of the variables after model averaging of the top $2AIC_c$ models. Saturated models included all variables that differed significantly among sibships: growth, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), malondialdehyde (MDA), and granulocyte-to-lymphocyte (G:L) ratio. We also tabulate the proportion of the variance explained by the best model (R^2), which includes all the variables that were restricted after model averaging, as well as the delta values (differences in AIC) of this model to the saturated model and to the null model (i.e. only including the intercept).

Development (pond drying)	Estimate	Unconditional SE	Relative importance
(Intercept)	-74.24	15.003	
growth	3.453	9.771	0.19
GPx	3.386	9.683	0.18
MDA	-2.306	8.12	0.15
CAT	-1.999	7.623	0.14
best model	Adjusted-R^2	delta to saturated model	delta to null model
~growth + GPx + MDA + CAT	-0.002276	2.1875	-3.5091
Development (predator)	Estimate	Unconditional SE	Relative importance
(Intercept)	25.29	14.42	
GPx	32.73	14.79	1
best model	Adjusted-R^2	delta to saturated model	delta to null model
~ GPx	0.1701	5.9034	2.8104
Growth (pond drying)	Estimate	Unconditional SE	Relative importance
(Intercept)	-6.75E-04	1.26E-04	
growth	-1.57E-04	1.80E-04	0.6
MDA	-3.81E-05	1.14E-04	0.18
best model	Adjusted-R^2	delta to saturated model	delta to null model
~ growth + MDA	0.1023	1.8246	-1.5631
Growth (predators)	Estimate	Unconditional SE	Relative importance
(Intercept)	2.10E-04	8.09E-05	
G:L ratio	1.12E-04	1.02E-04	0.67

best model	Adjusted-R^2	delta to saturated model	delta to null model
~ G:L ratio	0.1461	7.6836	2.2386
Morphology - RW2 (predator)	Estimate	Unconditional SE	Relative importance
(Intercept)	5.45E-03	1.48E-03	
SOD	1.19E-03	1.60E-03	0.49
GPx	0.0007754	0.0013543	0.38

best model	Adjusted-R^2	delta to saturated model	delta to null model
~ SOD + GPx	0.1247	6.1053	0.8889
Morphology - RW3 (predator)	Estimate	Unconditional SE	Relative importance
(Intercept)	4.71E-03	1.11E-03	
GPx	0.003759	0.001135	1

best model	Adjusted-R^2	delta to saturated model	delta to null model
~ GPx	0.3444	5.8217	7.5244
Morphology - RW4 (pond drying)	Estimate	Unconditional SE	Relative importance
(Intercept)	2.13E-03	1.10E-03	
MDA	0.0027807	0.0024722	0.7
SOD	0.001689	0.0016539	0.61
GPx	-0.000434	0.0010228	0.4
CAT	-0.0013608	0.0022856	0.31
G:Lratio	0.0004532	0.0010136	0.22
growth	-0.0010814	0.0015693	0.19

best model	Adjusted-R^2	delta to saturated model	delta to null model
~ MDA + SOD + GPx + CAT + G:Lratio + growth	0.3394	0	3.8806
Morphology - RW4 (predator)	Estimate	Unconditional SE	Relative importance
(Intercept)	-5.33E-04	1.38E-03	
MDA	0.0052396	0.0018881	1

GPx	0.0025341	0.0022887	0.79
CAT	-0.0008726	0.0018844	0.28
best model	Adjusted-R^2	delta to saturated model	delta to null model
~ MDA + GPx + CAT	0.4071	3.5205	7.8918

Trade-offs of plasticity

We evaluated trade-offs between plasticity elicited by different factors only when we observed significant differences among sibships for that trait in response to both pond drying and predator cues. Sibships with high developmental plasticity against pond drying could not delay development when exposed to predators ($F_{1,18}$ = 8.514, P = 0.0092, r^2 = 0.57; Figure 1), and *vice versa.* However, we found no significant relationship between growth plasticity in response to pond drying and in response to predator cues ($F_{1,18}$ = 0.790, P = 0.3859, r^2 = 0.20; see Additional File 3) or in morphological plasticity (RW4; $F_{1,18}$ = 1.413, P = 0.2499, coefficient of correlation = 0.27; see Additional File 3).

Discussion

The existence of maintenance costs of plasticity has been proposed as one of the main causes for within-population variation in the degree of plasticity (DeWitt, 1998; Lande, 2014; Chevin and Lande, 2015). However, only few studies have empirically detected such costs (reviewed in Van Buskirk and Steiner, 2009; Auld et al. 2010). In part it could be that maintenance costs of plasticity are not as important as previously thought in limiting the evolution of phenotypic plasticity (Murren et al. 2015), or that selection acts to reduce them (Auld et al. 2010). It could

also be that previous studies have often sought costs in terms of rather broad differences in key traits such as body condition or growth, which bear a strong direct effect on fitness and are therefore likely to have been eroded by selection. Surprisingly, despite recurrent arguments in the literature regarding likely energetic expenses of maintaining active sensory machinery to continuously track environmental variation, no studies to date have attempted to quantify maintenance costs of plasticity from a physiological or biochemical point of view (Auld et al. 2010).

Here we detected physiological maintenance costs associated with the ability of anuran larvae to alter their development, growth, and morphology in response to risk of pond drying and predator cues. We found no evidence for associations between degree of plasticity and metabolic rate or the amount of fat reserves, which we had initially expected to be good markers of costs if costs of plasticity were paid in terms of energetic currency. Instead, we found that the degree of phenotypic plasticity was consistently associated with higher levels of oxidative stress and, to a lesser extent, challenged immunological state.

GPx activity was strongly and positively associated with plasticity in developmental rate and growth rate in response to both pond drying and predator presence, and also with predator induced morphological changes. Catalytic function of GPx consists in reducing free hydrogen peroxide (H_2O_2) to water, thus protecting cells from oxidative damage. Increased GPx activity indicates H_2O_2 overproduction, which is a clear indication of enhanced mitochondrial respiration (Murphy, 2009). H_2O_2 plays several important roles at the cellular level, especially in terms of ageing regulation so that intracellular increases in H_2O_2 are genetically regulated to increase in order to induce cell death (Sedensky and Morgan, 2006; Giorgio et al. 2007). In contrast, induced overexpression of antioxidant enzymes seems to extend the organism's lifespan (Landis and Tower, 2005; Schriner et al. 2005). Nevertheless, the

mechanisms of such lifespan extension are not yet clear and significant questions remain (Lu and Finkel, 2008; Liochev, 2013), especially since reduced antioxidant activity has also been described to extend lifespan via increased sensitivity to apoptosis (Ran et al. 2007).

In addition to GPx, the activity of another antioxidant enzyme, SOD, and MDA concentration -the principal product of polyunsaturated fatty acid peroxidation- were strongly and consistently associated with predator induced phenotypic plasticity in our spadefoot toad larvae, further supporting the idea that plasticity maintenance incur in costs of increased oxidative stress. The enzyme SOD catalyzes the dismutation of the superoxide O_2^- into either oxygen (O_2) or H_2O_2, and is therefore also essential in protecting cells from oxidative stress and probably also contributing to lifespan regulation (Sun et al. 2002; Landis and Tower, 2005). In turn, MDA concentration denotes the prevalence of free radicals in tissues, hence indicating the extent of lipid peroxidation resulting from oxidative stress (Hulbert et al. 2007). Excess lipid peroxidation usually varies membrane composition and affects biological macromolecules like DNA, thus compromising cell stability and inflicting irreversible damages leading to aging (Hulbert et al. 2007). Our results conclusively show that high plastic genotypes are associated with elevated production of free radicals, as indicated by their higher levels of antioxidant activity and lipid peroxidation. Overproduction of free radicals is caused by enhanced metabolism (De Block and Stoks, 2008; Monaghan et al. 2009), and the fact that we found evidence for higher oxidative stress but not higher metabolism itself suggests that our biochemical assays to determine activity of antioxidant enzymes and lipid peroxidation were more sensitive and had more discriminatory power than our measures of metabolic rate to discern subtle differences among sibships under benign conditions (full water volume, *add libitum* food and lack of predator cues). We have found metabolic costs associated with actual production

of adaptive plastic changes in larval amphibians, as when newts increase their dark pigmentation in low albedo environments (Polo-Cavia and Gomez-Mestre, 2017); or spadefoot toad larvae accelerate development in response to pond drying to trigger an early metamorphosis (Kulkarni et al. 2017). In both cases, however, the production of induced phenotypic changes required either pigment production or a great deal of organogenesis, requiring substantial shifts in metabolism that we managed to measure. Maintenance costs of plasticity, however, would be much more subtle than production costs, as they would be limited to the energy devoted to sensing the environment and 'keeping ready' the neuroendocrine and genetic mechanisms enabling phenotypic changes.

We also found that greater plasticity in growth in response to predator cues was also associated with increased granulocyte-to-lymphocyte ratio. The granulocyte-to-lymphocyte ratio usually increases under stressful scenarios and is mediated by glucocorticoid secretion (Davis et al. 2008; Burraco et al. 2017). Increased granulocyte-to-lymphocyte (or neutrophil/heterophil to lymphocyte) ratio is also related to increased susceptibility to diseases (Huff et al. 2005; Duffy et al. 2006) and to poor body condition or health (Gomez et al. 2008; Lobato et al. 2009). Depressed immunological function and increased susceptibility to infections have been observed to be a consequence of phenotypic alteration in amphibians, i.e. production costs of plasticity. Thus, tadpoles forced to accelerate development to avoid pond drying reduced their immune-function in the juvenile phase (Gervasi and Foufopoulos, 2008). However, to date there was no evidence that maintenance of plasticity itself could take a toll on the immune status so that more plastic genotypes showed lower defenses.

We also found an intriguing trade-off between adaptive developmental plasticity to pond drying and to predator cues in spadefoot toad larvae. Sibships that accelerated development in response to pond dry-

ing failed to delay metamorphosis when exposed to predator cues. Many amphibian species have complex life cycles with an aquatic larval phase that grows in temporary ponds and thus are often at risk of desiccation. The ability to induce rapid morphogenesis and to accelerate development when water levels drop, results adaptive (Denver et al. 1998). Predators also pose serious risks for larval survival, but amphibian larvae increase their survival odds in the presence of predators by lowering their activity rate and metabolism (Relyea, 2004; Barry and Syal, 2013), which often results in slowed development (Laurila et al. 2004). The trade-off found here suggests that the evolution of adaptive developmental plasticity against one of these environmental risks may be hampered by selection posed by the other factor, especially since both developmental responses (accelerated and delayed metamorphosis) are mediated by the same neuroendocrine pathway (Hossie et al. 2010; Maher et al. 2013; Gomez-Mestre et al. 2013). However, we have not directly addressed here the combination of these environmental inputs and further studies combining them would be required to elucidate the importance of this trade-off.

In conclusion, developmental, growth, or morphological plasticity against two common risks for amphibian larvae showed physiological maintenance costs such that more plastic genotypes (sibships) constitutively incurred in higher oxidative stress and in some cases worse immune state even under benign conditions. In particular, higher plasticity was associated with elevated antioxidant enzymes activity, lipid peroxidation, and granulocyte-to-lymphocyte ratio. As far as we know, this is the first physiological assessment of maintenance costs of plasticity. Our results suggest that dismissal of maintenance costs as an important factor in the evolution of adaptive plasticity may be premature, in agreement with many theoretical studies (Chevin et al. 2010;

Gomez-Mestre and Jovani 2013; Scheiner, 2016). More studies linking the degree of plasticity to oxidative stress, fitness and lifespan are needed.

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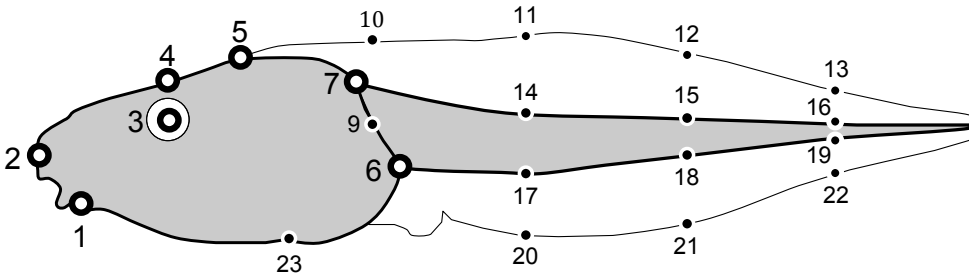
Additional File 1



Landmarks

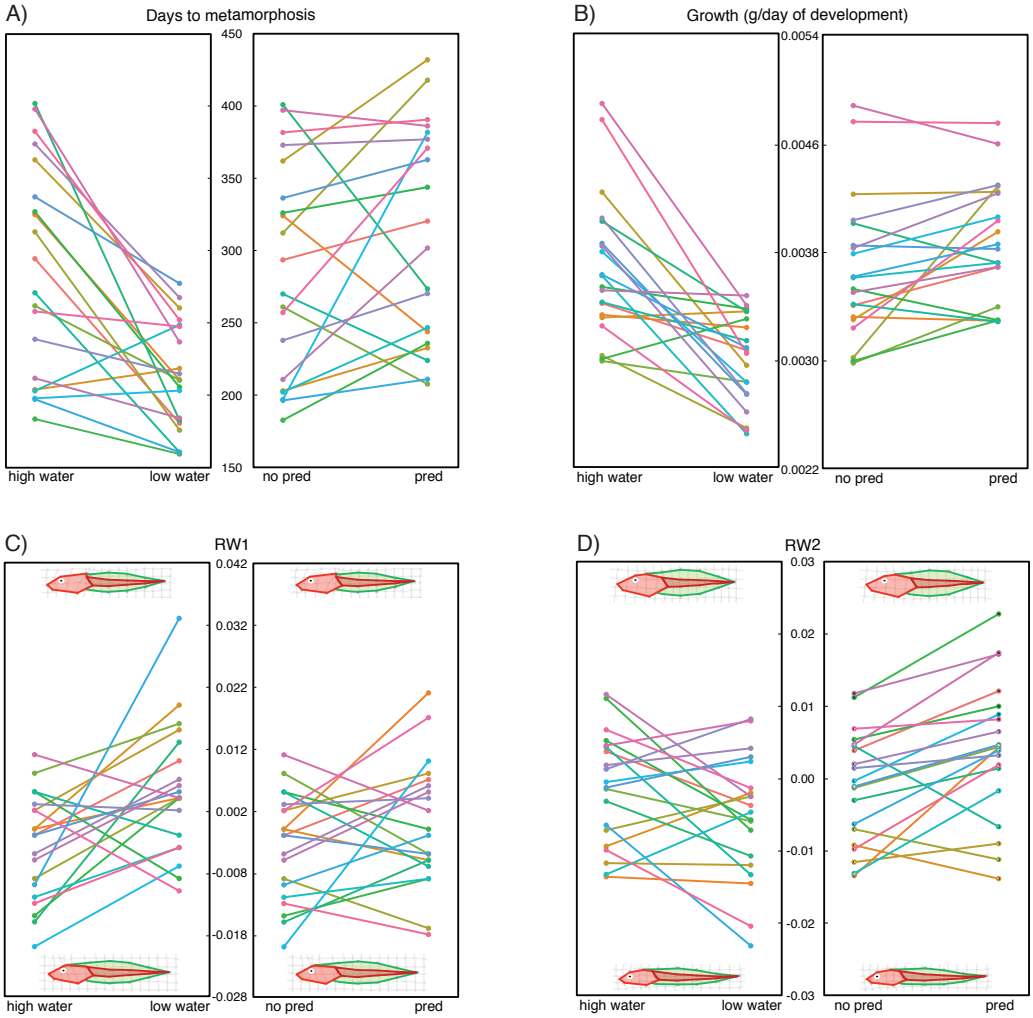
1: Margin of lower lip.
2: Margin of upper lip.
3: Eye.
4: Upper edge perpendicular to eye.
5: Dorsal fin insertion .
6: Body-tail muscle insertion at the lower end.
7: Body-tail muscle insertion at the upper end.
8: Tip of tail.

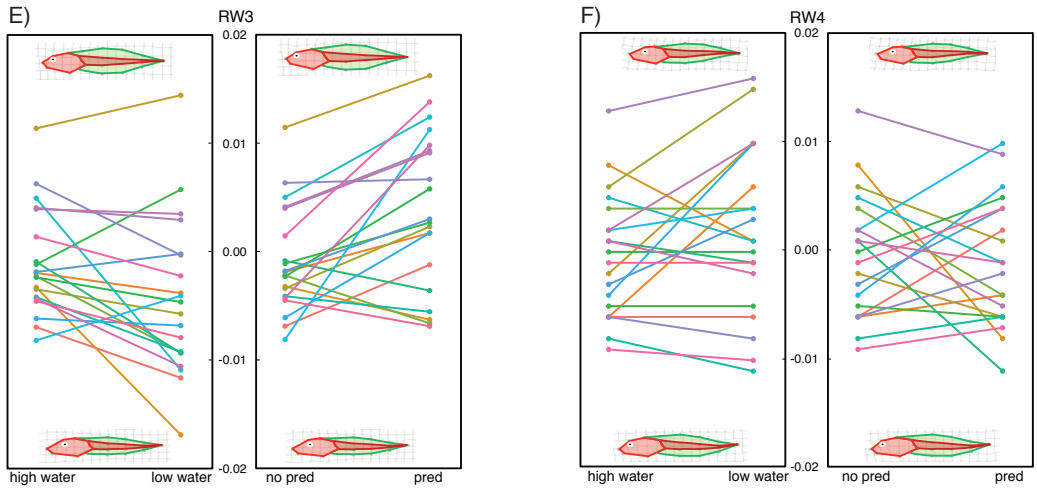
- **Semilandmarks**

9: Junction between body and tail muscle line.
10: Upper edge of tail fin perpendicular to 9.
11-13. Upper edge of tail fin perpendicular at 1/4, 2/4, and 3/4 between 8-9.
14-16: Upper edge of tail muscle perpendicular at 1/4, 2/4, and 3/4 between 8-9.
17-19: Ventral edge of tail fin perpendicular at 1/4, 2/4, and 3/4 between 8-9.
20-22: Ventral edge of tail muscle perpendicular at 1/4, 2/4, and 3/4 between 8-9.
23: Ventral body perpendicular at 3/4 between 2-6.

Additional file 1. Landmarks and semilandmarks used for morphometric analyses.

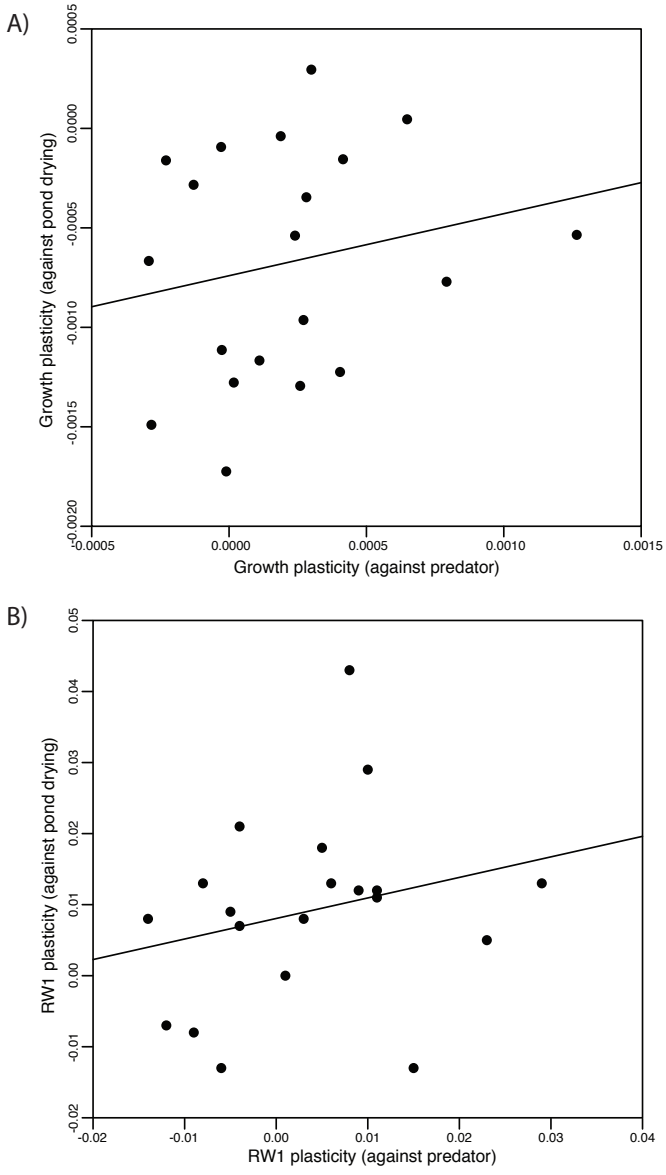
Additional File 2

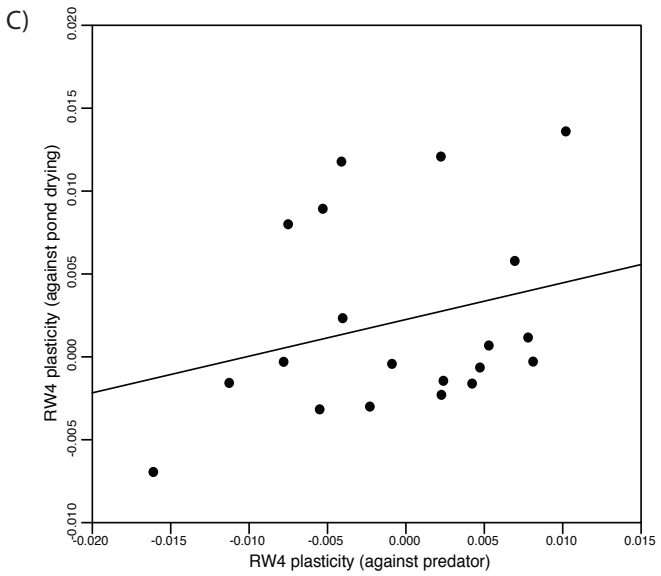




Additional File 2. Plasticity reaction norms of 20 *Pelobates cultripes* sibships responding to pond drying (left side of each graph) or predator presence (right side of each graph) in terms of developmental (A), growth (B), and morphological (relative warps 1 to 4; C, D, E, F, respectively) plasticity. Transformation grid in C, D, E, and F graphs indicate the direction of variation of each landmark.

Additional File 3





Additional File 3. Correlations of plastic responses against pond drying (Y-axis) and predators (X-axis) in growth (A) and morphology (B and C; in the relative warps –RW- 1 and 4, respectively).

CHAPTER 4

Physiological mechanisms of adaptive developmental plasticity in *Rana temporaria* island populations

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Abstract

Adaptive plasticity is essential for many species to cope with environmental heterogeneity. In particular, developmental plasticity allows organisms with complex life cycles to adaptively adjust the timing of ontogenetic switch points. Size and timing of metamorphosis are reliable fitness indicators in organisms with complex cycles. However, environmental inputs can affect the degree of their adaptive developmental plasticity, which may also imply short and long-term consequences. The physiological machinery of developmental plasticity commonly involves neuroendocrine activations that can imply metabolic alterations. Nevertheless, we have still incomplete knowledge about how these mechanisms evolve under environments that selects for differences in adaptive plasticity. In this study, we investigate the physiological mechanisms underlying divergent degrees of developmental plasticity across *Rana temporaria* island populations inhabiting different types of pools in northern Sweden. In a laboratory experiment we estimated developmental plasticity of amphibian larvae from six populations coming from three different island habitats: islands with only permanent pools, islands with only ephemeral pools, and islands with a mixture of both types of pools. We exposed larvae of each population either to constant *water level* or simulated pool drying and estimated the physiological consequences in terms

of corticosterone levels, oxidative stress, and telomere length. We found that populations from islands with only temporary pools had a higher degree of developmental plasticity than those from the other two types of habitats. All populations increased their corticosterone levels to a similar extent when subjected to simulated pool drying, and therefore it does not explain differences among populations. However, tadpoles from islands with temporary pools showed lower constitutive activities of catalase and glutathione reductase, and also showed overall shorter telomeres. The observed differences are indicative of physiological costs of increased developmental plasticity, suggesting that the potential for plasticity is constrained by its maintenance costs. Thus, high levels of responsiveness in the developmental rate of tadpoles have evolved in islands with pools at high but variable risk of desiccation. Moreover, the physiological alterations observed may have important consequences for short-term survival and in the long term on lifespan itself.

Keywords: Amphibians; Corticosterone; Developmental plasticity; Evolutionary physiology; Oxidative stress; Telomere length

Introduction

Adaptive phenotypic plasticity is an effective mechanism to cope with changing environments (Snell-Rood, 2013; Chevin and Lande, 2013; Murren et al. 2015). It increases population viability and facilitates the maintenance of genetic variation in two main ways: reducing the effect of genetic drift by moderating bottlenecks; and shielding genetic variants from selection (Dragui and Whitlock, 2012; Gomez-Mestre and Jovani, 2013). This shielding effect may slow down the response to selection (Price et al. 2003), but at the same time increases the odds of population persistence under environmental heterogeneity and preserves genetic variation that grants a higher level of adaptive potential (Price et al. 2003; Gomez-Mestre and Jovani, 2013). Because plasticity confers a higher capacity for surviving new or rapidly changing environments, it can facilitate divergence among populations (Levin, 2009; Snell-Rood et al. 2010; Svanbäck and Schluter, 2012) and ultimately foster speciation (Pfenning et al. 2010; Dragui and Whitlock, 2012).

Environmental heterogeneity affects the degree and the range of adaptive plasticity (Lind and Johansson, 2007; Gomez-Mestre and Jovani, 2013; Chevin and Lande, 2015). Heterogeneous environments are expected to favor highly plastic genotypes whereas homogeneous environments would tend to reduce plasticity (Lind and

Johansson, 2007; Beldade et al. 2011; Schlichting and Wund, 2014). Adaptive divergence in the plastic responses of populations evolving under different environmental regimes requires qualitative or quantitative population divergence in the mechanisms underlying phenotypic expression. The expression of alternative phenotypes relies on changes in gene expression (Schlichting and Wund, 2014) and/or changes in the regulation of physiological pathways (Ricklefs and Wikelski, 2002). Thus, understanding the physiological mechanisms regulating developmental plasticity is key in understanding the origin of evolved differences among populations in contrasting environments.

Developmental plasticity is particularly critical for amphibians because they are typically species with low vagility and high philopatry to highly variable habitats (Smith and Green, 2005). The majority of amphibians exhibit ancestral aquatic reproduction (Gomez-Mestre et al. 2012) and breed in temporary water bodies where the larvae develop until metamorphosis. A major larval-stage risk is pond drying and amphibian larvae can often detect fluctuations in water level and accelerate development in response to decreased water levels to achieve an early metamorphosis (Denver, 1997; Ritcher-Boix et al. 2006). Theory would predict that populations exposed to more fluctuating hydroperiods would exhibit greater developmental plasticity. The populations of *Rana temporaria* on the Swedish islands is a paradigmatic example of the relationship between environmental heterogeneity and degree of adaptive plasticity (Lind and Johansson, 2007). These populations were established between 70 and 800 years ago (Johansson et al. 2005) by frogs migrating from the mainland (Lind et al. 2011). The islands have rocky pools where the frogs breed. These pools vary greatly and consistently in depth and size among islands, and consequently vary in average pond duration,

ranging from permanent to ephemeral (Lind and Johansson, 2007). Over the last decade, several studies have analyzed the adaptive divergence in developmental rate among these *R. temporaria* populations. These studies have found signs of developmental canalization so that populations occupying islands with only ephemeral pools show overall faster developmental rates than populations from islands with permanent pools (Lind et al. 2008) while also showing reduced within-population genetic variation for developmental rate (Lind et al. 2008). Moreover, island populations show marked differences in plasticity of their developmental rate (Lind and Johansson, 2011) according to pond duration during the breeding season. In addition, Lind and Johansson (2009) found evidence of costs of developmental plasticity, although these were only noticeable for the most plastic populations. Faster developing populations were also found to express higher levels of thyroid hormone receptors (alpha and beta) and genes associated with higher metabolic activity (Johansson et al. 2013). This is congruent with mechanisms of developmental acceleration found in other species, namely increased thyroid hormone and corticosterone levels (Gomez-Mestre et al. 2013).

Developmental acceleration in amphibians is mediated by neuroendocrine pathways, especially by the hypothalamic pituitary adrenal axis (HPA) (Denver, 2009). The HPA-axis is activated by external environmental inputs such as water height (Boorse and Denver, 2003), ultimately resulting in increased corticosterone production, which together with thyroid hormone activate or repress synergistically nuclear target genes causing increased metabolic rate and accelerated morphogenesis (Denver, 2009). However, prolonged corticosterone secretion accelerates metabolism (Wack et al. 2012) but at the cost of overproduction of toxic substances called reactive oxygen species (ROS) that can inflict considerable cellular damage. Cellular oxidative

damage occurs when the production of ROS is greater than the detoxifying capacity of antioxidant enzymes (Apel and Hirt, 2004). Since the *R. temporaria* populations studied by Lind and Johansson (2007) show adaptive differences in developmental rate and vary in the regulation of genes associated with metabolic activity, we hypothesized that populations would differ according to habitat type in their corticosterone regulation of development and in the level of oxidative stress experienced.

High ROS production also results in DNA damage, of which telomere shortening is of great importance because of its association with life-history trade-offs and lifespan (Von Zglinicki, 2002; Monaghan et al. 2009). Telomeres are non-coding tandem repeat sequences of the terminal regions of the chromosomes with high G-C strand asymmetry (Blackburn, 1991). Telomeres are determinants of cell senescence and are also involved in chromosome stability by avoiding chromosome fusion (O'Sullivan and Karlseder, 2010). Telomere replication occurs via reverse transcriptase telomerase that adds telomeric repeats to 3' overhang. However, telomere ends shorten over time after each cell division until critical telomere length is reached and initiates apoptosis, or programmed cell death (Campisi, 2003). Enhanced telomere abrasion has been described as a consequence of compensatory growth and developmental acceleration (Metcalfe and Monaghan, 2011), mainly as a direct consequence of oxidative damage. In the wild, telomere shortening studies are principally focused on ageing (Hausmann and Vleck, 2002; Bize et al. 2009) and body condition quantification (Trusina, 2014; Boonekamp et al. 2014). However, only a few evolutionary studies include telomeres as a mechanism under selection even though telomere shortening correlates with a large number variations in life-history traits (Haussmann and Marchetto, 2010), and most of these studies have been mostly conducted on humans (Seluanov et al. 2007; Gorbunova and Seluanov, 2009; Eisenberg, 2011).

Here we examine whether adaptive differences in developmental rate among *R. temporaria* populations are associated with changes in corticosterone levels, oxidative stress, or telomere length. We hypothesize that populations experiencing high developmental plasticity will pay a cost in terms of high constitutive levels of corticosterone and oxidative stress, and shorter telomeres, compared with less plastic populations.

Material and methods

Study area and field sampling

We studied the physiological consequences of adaptive divergence in developmental rate of *Rana temporaria* tadpoles from six islands located in the Gulf of Bothnia (Umea, Sweden) in a 10 km section of the coastline. The size range of the islands is between 9 and 38 ha. Frogs breed in water filled pools created by rocky depression on these islands. The pools on the islands differ in their water permanence such that some have ephemeral pools, other permanent pools, and others have a mixture of permanent and ephemeral pools. There is no relationship between predator abundance and life history traits among pools on these islands (Johansson et al. 2005), and consequently pond duration seems to be the main environmental factor determining larval development in this system.

On 5 May 2014 we collected a maximum of four clutches from each of six islands. We separated the islands into three type of habitats attending to their pool characteristics: two had only permanent pools (Storhaddingen 63º 40'N, 20º 25' E; Lillhaddingen 63º 40'N, 20º 24'E), two only ephemeral pools (Sävar-Tärnögen 63º 45'N, 20º 36'E; Ålgrundet 63º 41'N, 20º 25'E) and two a mixture of permanent

and ephemeral pools (Petlandsskär 63º 39'N, 20º 24'E; Bredskär 63º 39'N, 20º 18'E). Clutches were raised until Gosner stage 25 (Gosner, 1960) in 1 L plastic containers (9.5 cm x 9.5 cm, height 10 cm) filled with 500 mL of reconstituted soft water (see Räsänen et al. 2003 for details) in a climatic chamber at 14 ºC and a light: dark cycle of 18 h : 6 h simulating natural photoperiod conditions.

Experimental set-up

One week after egg sampling tadpoles reached the free-feeding stage and started to swim actively (Gosner stage 25). At that time, 12 tadpoles per clutch (six tadpoles per treatment) for a total of 216 experimental units were individually transferred to 1 L containers filled with 750 mL of reconstituted soft water, where they were raised until metamorphosis. We renewed water every 4 days and tadpoles were fed *ad libitum* with lightly boiled spinach at each day of water change (Richter-Boix et al. 2014). Containers were placed on shelves in a random order with respect to treatment and island in a walk-in climate chamber. *Te*mperature and light were set to 22º C and to 18 h : 6 h of light : dark. The experiment included a water level factor with two levels: constant water and simulating pool drying conditions. In the simulating pool drying treatment we decreased the initial water volume of 750 mL (10 cm) by 33 % at each water change starting on day 5 until day 25, keeping the water volume was constant at 66 mL (1 cm depth) afterwards. Water temperature did not differ significantly between treatments despite the differences in water level. The experiment ended when tadpoles reached Gosner stage 42 (front legs visible). We monitored tadpoles twice a day (09.00 and 21.00) to check for metamorphs. At this stage tadpoles were photographed to determine their length using ImageJ (version 1.47t), and were wei-

ghed in a high precision balance. Clutch size from each island population varied between 2 and 4.

Tissue collecting

Tadpoles at 42 Gosner were euthanized by immersion in a buffered solution of MS-222. A portion of muscle from the tadpoles' tail was removed with a surgical blade and preserved at -20 ºC for telomere analyses. The rest of the tail was snap frozen in liquid nitrogen and preserved at -80 ºC until corticosterone assays were conducted. The rest of the body was also snap frozen for oxidative stress assays.

Corticosterone assay

Corticosterone content was determined in tails (50-60 mg) collected from tadpoles at 42 Gosner Stage. Tissue was homogenized with an Ultraturrax TP18/10 (Hanke & Kunkel; IKA-Werk) during 30 s and the hormone was extracted following an organic phase extraction with 1 mL ethyl acetate during 30 min at 4 °C and continuous shaking. Samples were then centrifuged at 5000 rpm during 15 min and a known volume of the supernatant was taken and evaporated in a speedVac. Dried elutes were re-suspended in a final volume of 120 mL of the assay buffer provided with the enzymoinmunoassay kit supplemented with ethanol (< 5 % of final volume) to aid the re-suspension of steroids. We assayed each sample in duplicate (50 mL per sample) for corticosterone determination through specific enzymoinmunoassays (Arbor Assays, K014-H1/H5). The corticosterone antibody has low cross-reactivities to cortisol (0.38 %), 11-desoxycorticosterone (12.3 %), or progesterone (0.24 %). The efficiency of the extraction was checked by spiking several aliquoted samples

with 100 pg of exogenous corticosterone prior to extraction and comparing them to the non-spiked aliquotes. Recovery of exogenous corticosterone was never lower than 96 %. The lowest point in the corticosterone standard curve was 2.29 pg/mL. To test for assay precision and variability, we determined the coefficient of variation (CV %) for intra- and inter-assay variation. Intra-assay variation was 8.74 %. Inter-assay variation was 13.23 % in the highest point of the standard curve and and 6.86 % in the lowest point (n = 8 assays in both cases). Corticosterone concentration was calculated from the % B/B0 curve by using the 4PLC fitting routine and following the online tool from http://www.myassays.com/arbor-assays-corticosterone-enzyme-immunoassay-kit.assay (Accessed 20 April 2017).

Oxidative Stress assay

We quantified the activity of three antioxidant enzymes (catalase, glutathione peroxidase, and glutathione reductase). We also quantified malondialdehyde (MDA) concentration, a product formed during lipid peroxidation, total glutathione (GSHt) and the ratio of oxidized to reduced glutathione (GSH/GSSG ratio). After evisceration, tadpoles were individually homogenized with a Miccra homogenizer (Miccra D-1) at 35,000 rpm in a buffered solution to inhibit proteolysis (1:4; w:v; Burraco et al. 2016). The homogenates were centrifuged at 14,000 rpm for 30 min at 4 ºC and the resulting supernatants were aliquoted into six 0.6 mL tubes and cryopreserved at -80 ºC. We determined total protein content by standard Bradford's procedure (Bradford, 1976). The coefficient of variation in total protein determinations between duplicated samples was on average 4.27 %.

Catalase (CAT) catalytic activity was indirectly quantified following Cohen and Somerson (1969). According to this protocol, potassium

permanganate ($KMnO_4$) was used as an oxidizing agent that reacts with the catalase substrate hydrogen peroxide (H_2O_2) giving a red color that can be read at 480 nm 5 min after KMnO4 was added. We used commercial catalase (SIGMA – 60634) for standard curves preparation. The coefficient of variation between duplicated samples was on average 3.26 %. Glutathione peroxidase (GPx) activity was quantified following the protocol developed by Paglia and Valentine (1967). An excess of glutathione reductase (GR) reduces continually with NADPH the oxidized glutathione (GSSG) producing a constant level of reduced glutathione (GSH). We estimated NADPH oxidation spectrophotometrically at a wavelength of 340 nm. The coefficient of variation between duplicated samples was on average 2.12 %. GR activity was determined assessing the decrease in absorbance at 340 nm due to NADPH oxidation (Cribb et al. 1989), and assays showed an average coefficient of variation between duplicated samples of 2.74 %. Lipid peroxidation was determined according to Buege and Aust (1978) by measuring MDA concentration. MDA is one product of the lipid peroxidation that reacts with thiobarbituric acids, generating a red product that absorbs at 535 nm. We quantified MDA concentration in nmol / mL by measuring optical density of each sample and then subtracting the blank values and comparing with the calibration curve. The coefficient of variation between duplicated samples was on average 3.93 %. Total glutathione (GSHt) was determined following Galván et al. (2010). Homogenates were diluted (1:10, w:v) and homogenized in a stock buffer solution (0.01 M PBS and 0.02 M EDTA). Three working solutions were prepared from the same stock buffer as follows: (a) 0.03 mM of NADPH, (b) 6 mM 5,5'-Dithiobis(2-nitrobenzoic acid) (DNTB), and (c) 50 units of GR/mL. Solution a and b were mixed using a ratio of 7:1 (A:B) and then 160 µL of this mixture was added to 40 µL of supernatant. After

15 seconds, 20 µL of the solution C were added and absorbance was read at 405 nm after 30 and 60 seconds. Total concentration of glutathione was determined by comparing changes in absorbance between the two readings, according to a standard curve generated by serial dilutions of glutathione from 1 nM to 0.031 nM. The repeatability was 90.3 % (n = 10 samples).

Relative telomere length quantification

Genomic DNA for telomere measurements was isolated using a high-salt DNA extraction protocol on a portion of tail muscle. All determinations were made from muscle tissue to avoid confounding differences between tissues in telomere length (Fiedrich et al. 2000; Dlouha et al. 2014). Relative telomere length assays were performed using quantitative PCR (qPCR) and calculated as the ratio of telomere repeats to a single-copy gene (T/S ratio; Cawthon, 2002). As a single copy gene we used glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GenBank accession no. AF255390). Forward and reverse sequences of primers used to amplify GAPDH were 5-AACCAGCCAAGTACGATGACAT-3' (GAPDH-F) and 5'-CCATCAGCAGCAGCCTTCA-3' (GAPDH-R), respectively. Forward and reverse primers of the target gene were 5'CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3' (Tel1b) and 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3' (Tel2b), respectively. We performed qPCR in two separated plates for GAPDH and telomere genes by adding 20 ng of genomic DNA from each sample. The set of primers used (Tel1b/Tel2b and GAPDH-F/GAPDH-R) was at an initial concentration of 900 nM containing 10 µL of Brilliant SYBR Green QPCR Master Mix (Roche) in a final volume of 20 µL. PCR protocol consisted of 10 min at 95 ºC followed by 30 cycles of 1 min at 56 ºC and 1 min at 95 ºC for

telomere fragment amplification, and 10 min at 95 ºC followed by 40 cycles of 1 min at 60 ºC and 1 min at 95 ºC for GADPH fragment. We conducted qPCRs on a LightCycler 480 (Roche) and we tested the efficiency of each plate by performing a control standard curve by serially diluting a pool of samples from the different treatments and islands in triplicate (160, 40, 10, 2.5 and 0.66 ng of DNA per well). We calculated the cycle threshold (Ct) value of each sample for each plate. Threshold is the basal level of fluorescence. Ct is defined as the number of cycles needed to detect a signal above the threshold. All samples were run in duplicate and relative telomere length was calculated following the formula (Plaffl, 2001):

$$\text{ratio} = [(E_{\text{telomere}})^{\Delta Ct\ \text{telomere}\ (\text{control} - \text{sample})}] / [(E_{\text{GAPDH}})^{\Delta Ct\ \text{GAPDH}\ (\text{control} - \text{sample})}]$$

where E_{telomere} is the qPCR efficiency of telomere fragment; E_{GAPDH} is the qPCR efficiency of the GAPDH fragment; ΔCt telomere is the Ct deviation of control – sample of the telomere (target gene) fragment; ΔCt GAPDH is the Ct deviation of control – sample of reference of GADPH (gene of reference) fragment. The coefficient of variation for duplicated samples was 0.65 % on average for GAPDH assays and 1.36 % for telomere assays.

Statistical analyses

Statistical analyses were conducted in R, version 3.3.1 (R Development Core Team). We checked whether residuals followed normal distributions conducting Kolgomorov-Smirnov tests ("lillie.test" in "nortest" package, version 1.0-2). We also tested for homoscedasticity using Barlett's tests with the function "bartlett.test" ("car" package; version 2.0-22). We fitted linear and generalized mixed effect models using "lmer" function for normal data and "glmer" for non-normal data (package "*lme4*"; version 1.1-7). We used *water*

level (constant or simulated pool drying) and island habitat as fixed factors, and included *clutch* nested within *island* as random factors in the models. We used likelihood ratio tests to determine the significance of each predictor. We modeled with a Gaussian distribution normally distributed data. Days to metamorphosis, corticosterone, GPx, GR, GSH, and telomere data were log-transformed to meet parametric assumptions. We estimated growth rate as log (body mass at 42 Gosner stage) – log (days to reach 42 Gosner stage).

Results

Survival was very high throughout the experiment (93.05 %) and we found no significant differences between treatments, clutches, or island habitats.

Simulated pool-drying induced accelerated development in *R. temporaria* larvae (χ^2 = 58.05, $P < 0.001$; Figure 1A), but the degree of response differed among island habitats as indicated by a significant island habitat by water level interaction (χ^2 = 6.73, P = 0.035; Figure 1A). Tadpoles from islands with only ephemeral pools showed the highest developmental plasticity since they reduced the time to metamorphosis by 8.13 % on average, compared to tadpoles from islands with both type of pool-drying regimes and with only permanent pools, which accelerated their development by 4.40 % and by 4.57 % on average, respectively. Also, pool drying decreased larval growth rate by an average of 12.03 (χ^2 = 42.39, $P < 0.001$; Figure 1B). However, we did not find differences in growth rate among island habitats (χ^2 = 0.49, P = 0.784) or in the interaction between island habitat and water level (χ^2 = 1.99, P = 0.390; Fig 1B).

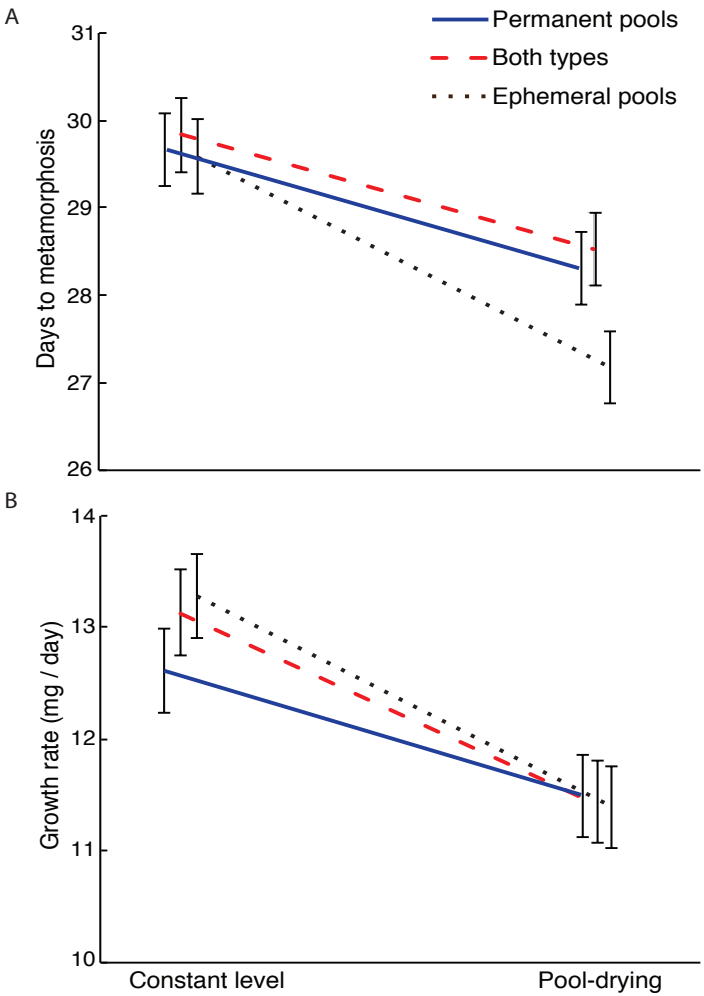

Figure 1. The effect of pool drying on (A) larval period and (B) growth rate in *Rana temporaria* tadpoles from three island habitats: islands with only permanent pools (blue line), islands with a mixture of ephemeral and permanent pools (dashed red line), and islands with only temporary pools (dotted black line). Data are least square means ± standard error.

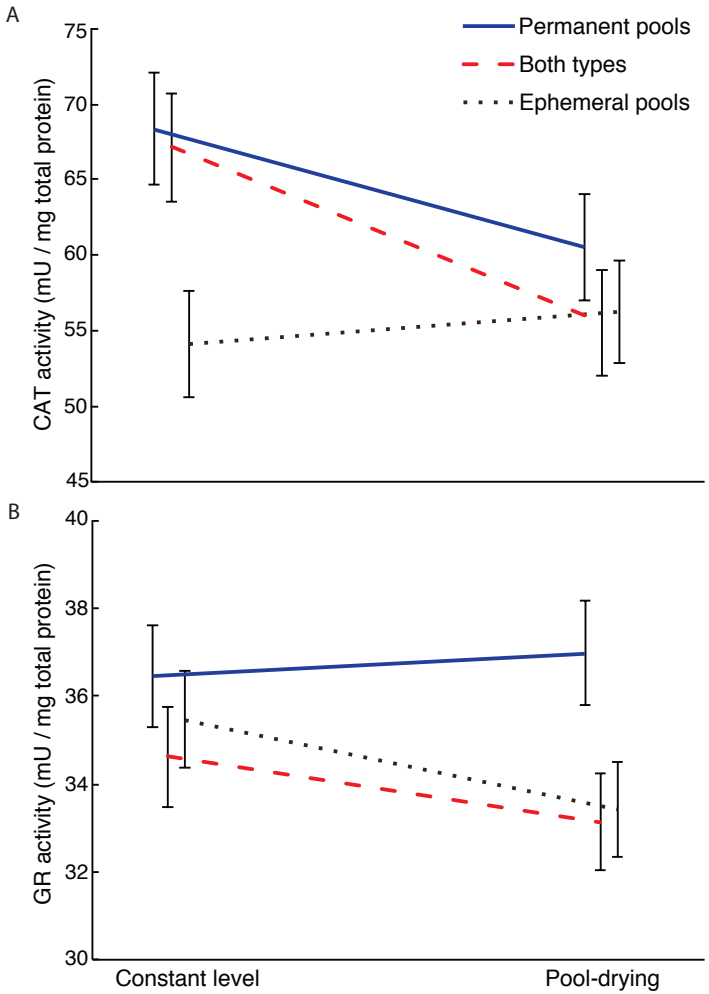

Figure 2. The effect of pool drying on (A) catalase (CAT) and (B) glutathione reductase (GR) activity on *Rana temporaria* tadpoles at 42 Gosner stage from three island habitats: islands with only permanent pools (blue line), islands with a mixture of ephemeral and permanent pools (dashed red line), and islands with only temporary pools (dotted black line). Data are least square means ± standard error.

Simulated pool desiccation induced endocrine responses in *R. temporaria* tadpoles, indicated by increased corticosterone levels by an average 25.48 % ($\chi^2 = 8.71$, $P = 0.003$; Figure I in Additional File 1). However, we did not find significant differences between island habitats or their interaction with water level (all $P > 0.110$; Figure I in Additional File 1).

We also found alterations in antioxidant responses of the different populations in relation to water level. CAT activity decreased by 9.14 % on average in tadpoles responding to pool drying ($\chi^2 = 3.84$, $P = 0.049$; Figure 2A). Island habitat also affected CAT activity ($\chi^2 = 6.56$, $P = 0.038$; Figure 2A) so that it was 11.21 % and 16.86 % lower in tadpoles from islands with ephemeral pools than in tadpoles from islands with both types of pools or with permanent pools, respectively. CAT levels were not affected by the interaction between pool drying and island habitat ($\chi^2 = 4.37$, $P = 0.112$; Figure 2A). Pool drying seemed to induce a slight increase in GPx levels, but the effect was not significant ($\chi^2 = 2.84$ $P = 0.092$; Figure II in Additional File 1). Neither island habitat nor its interaction with pool drying significantly affected GPx levels ($\chi^2 = 3.25$, $P = 0.197$, and $\chi^2 = 0.01$, $P = 0.996$, respectively; Figure II in Additional File 1). Pool drying did not alter GR levels ($\chi^2 = 2.371$, $P = 0.1236$; Figure 2B). However, we found marginally non-significant differences between island habitats ($\chi^2 = 5.778$, $P = 0.056$; Figure 2B). Tadpoles from islands with only ephemeral pools and from islands with both types of pools showed lower constitutive GR levels than tadpoles from permanent pools (6.26 % and 7.84 % lower GR levels on average, respectively). We did not find differences in the interaction between pool drying and island habitat in GR levels ($\chi^2 = 1.178$, $P = 0.555$; Figure 2B).

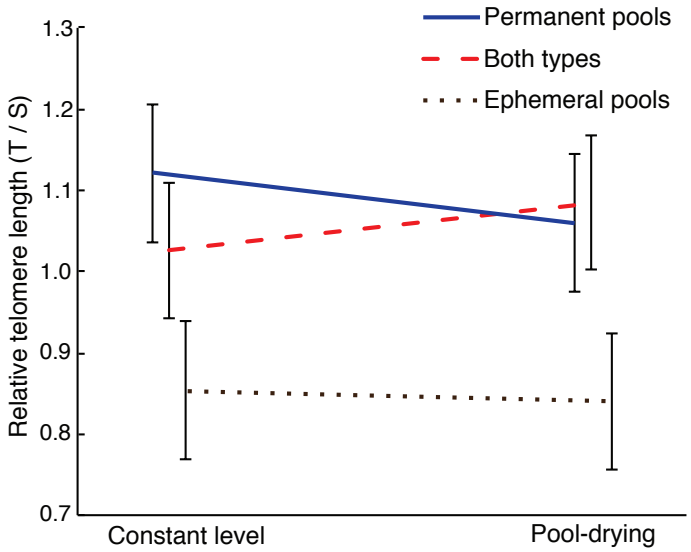

Figure 3. The effect of pool drying on relative telomere length (T/S ratio) of tail muscle tissue from *Rana temporaria* tadpoles at 42 Gosner stage from three island habitats: islands with only permanent pools (blue line), islands with a mixture of ephemeral and permanent pools (dashed red line), and islands with only temporary pools (dotted black line). Data are least square means ± standard error.

We found that alterations in antioxidant enzymatic activity were not necessarily associated with cellular oxidative damage, since MDA values were not affected by pool drying, and showed no differences among island habitats, or their interaction (all $P > 0.109$; Figure III in Additional File 1). Decreased water level resulted on average in 17.31 % lower GSH_t values ($\chi^2 = 9.49$, $P = 0.002$). Levels of GSH_t was unaltered by either island habitat or island habitat by water level interaction ($\chi^2 = 1.90$ $P = 0.386$ and $\chi^2 = 4.69$, $P = 0.096$, respectively; Figure IV in Additional File 1). The ratio of reduced to oxidized glutathione (GSH/GSSG ratio) was not affected by pool drying ($\chi^2 = 3.16$,

P = 0.076; Figure V in Additional File 1), and showed no differences among island habitats (χ^2 = 2.54, P = 0.281; Figure V in Additional File 1), or their interaction (χ^2 = 0.36, P = 0.833; Figure V in Additional File 1).

Relative telomere length was not affected by water level treatment (χ^2 = 0.010, P = 0.9227; Figure 3), but it varied significantly among island habitats (χ^2 = 6.077, P = 0.0479; Figure 3). Tadpoles from ephemeral pools showed on average 22.31 % shorter relative telomere length than tadpoles from permanent pools, and 19.89 % shorter relative telomere length than tadpoles from semi-permanent pools. The interaction between relative telomere length and water level was not significant (χ^2 = 1.459, P = 0.4822; Figure 3).

Relative telomere length and GSHt, did not correlate with duration of larval period or growth rate (all $P > 0.1098$). We found, however, a slight negative correlation between GSH/GSSG ratio and larval period (R^2 = 0.089; P = 0.019).

Discussion

Plasticity may foster adaptive divergence among populations exposed to different environmental regimes by allowing them to express alternative phenotypes for selection to act upon, which results in divergent evolutionary responses to selection (West-Eberhard, 2003; Lind and Johansson, 2007; Pfennig et al. 2010). Here we found evidence for divergence among *Rana temporaria* populations in their ability to accelerate development in response to decreased water level in accordance with the predominant pool drying regimes. The studied populations have been estimated to experience a small degree of neutral genetic differentiation (Lind et al. 2011), although *F*st estimates could have been biased

downwards due to high heterozygosity of the markers used (Lost, 2008; Edelaar et al. 2011) and hence population differentiation may be greater than previously thought. Populations from islands with only ephemeral pools showed greatest capacity for developmental acceleration in response to pool drying. Consequently, selection under different flooding regimes in these populations resulted in genetic accommodation of plasticity, not in canalization or genetic assimilation (West-Eberhard, 2003; Crispo, 2007). Selection for rapid larval development can result in canalized fast development and loss of plasticity (Gomez-Mestre and Buchholz, 2006; Johansson et al. 2013). In our study, populations inhabiting islands with ephemeral pools have evolved greater developmental plasticity. Our results differ somewhat from a previous study on this system (Lind and Johansson, 2007), where islands with both types of pools showed higher plasticity. This discrepancy can be a consequence of broader than expected interannual variability in pool duration (Lind and Johansson, 2007), among-population variation in costs of plasticity maintenance (Auld et al. 2010), and/or stochastic effects of sampling different genotypes over different years.

The mechanisms underlying developmental acceleration in amphibians are well known, and rely on activation of the hypothalamic-pituitary-axis (Denver, 1997; Denver, 2009). This neuroendocrine activation in amphibians results in higher thyroid hormone and corticosterone levels, which enhance cell replication rate (Haussmann and Marchetto, 2010) and morphogenesis (Denver, 2009). We observed that larvae from all populations increased corticosterone levels to a similar extent (around 25 %) when facing decreased water levels. Such up-regulation of corticosterone explains the ability of these populations to accelerate development, but does not reflect the observed among-population differences in their degree of plasticity. In contrast, the activity of antioxidant enzymes varied significantly

among populations, in agreement with their adaptive differences in developmental rate. This suggests that populations evolved metabolic differences resulting in different levels of ROS production or in different sensitivity to ROS during mitochondrial respiration (Vegiopoulos and Herzig, 2007). In this case, highly plastic *Rana* populations showed decreased constitutive levels of both CAT and GR enzymes. CAT transforms hydrogen peroxide to water and oxygen whereas GR reduces glutathione disulfide to the sulfhydryl form of glutathione, both processes being essential in protecting cells from oxidative damage. Low enzymatic levels are associated with exhaustion of the enzymes as a result of oxidative stress via toxic substances or ROS production (Barata et al. 2005; Srinivasan et al. 2007; Slos and Stoks, 2008). In addition, Johansson et al. (2013) found an increase in the gene transcript of catalase in tadpoles facing pool drying. Levels of catalase transcript were higher in populations that developed faster, supporting the idea of exhausted levels of these enzymes in populations with high developmental plasticity. Thus, lower activities of CAT and GR might indicate an enzymatic inactivation caused by an excess of ROS produced during developmental acceleration (D'Autréaux and Toledano, 2007; Monaghan et al. 2009). An alternative explanation might be that selection favored individuals that maximized mitochondrial respiration (Salin et al. 2015), hence producing less ROS when they experienced higher metabolic rates and showed lower antioxidant activity (Salin et al. 2015).

Telomere length varied among populations adapted to different pond-drying regimes by evolving different extents of developmental plasticity. This is a novel and intriguing finding and it may help to understand the implications of developmental plasticity to lifespan and fitness. Telomere shortening occurs after each cell replication so that telomeres usually shorten with age (Frenck et al. 1998; Asghar et al.

2015). However, telomere shortening is also linked to increased metabolism (Haussmann and Marchetto, 2010), particularly to oxidative stress originated during intense cell respiration (Monaghan et al. 2008; Haussmann and Marchetto, 2010). In wild populations, telomere shortening has also been described as a predictor of mortality (Barret et al. 2013), reproductive costs (Bauch et al. 2013) or the impact of infections (Asghar et al. 2016). Therefore, telomere shortening can result a reliable indicator of the biological age of individuals (Barret et al. 2013). In our experiment, antioxidant responses observed in *Rana* populations from islands with only ephemeral pools suggest a high ROS production derived from intense metabolism, which is associated with the attrition of telomeres. Thus, based on our oxidative stress and telomere length determinations, we would expect accelerated development to bear the consequence of reduced lifespan and hence possibly reduced fitness. Differences in telomere length among populations have been associated to trans-generational effects of male age at reproduction due to a progressive elongation of telomeres in sperm with age (Baird et al. 2006; Kimura et al. 2008; Eisenberg et al. 2012). Analogous maternal age effects have not been found (Kimura et al. 2008). Parental exposure to stressful conditions is also relevant to inheritance of telomere length, although these processes remain poorly understood (Haussmann and Heidinberg, 2015). In our study, shorter telomeres in tadpoles from islands with only ephemeral pools might be related to early age of first reproduction of males, which could be a long-term consequence of accelerated development against pool drying. However, further empirical studies will elucidate underlying mechanisms in telomere inheritance. Evaluations of telomerase activity and long-term studies testing the effects of parental telomere shortening on life-history traits of offspring will help to understand telomeric dynamic across generations.

Physiological differences among populations found in this study would indicate that metabolic costs exist associated to increased developmental plasticity. Such metabolic toll can compromise the health and lifespan of individuals, as indicated by shortening in the terminal chromosome regions. Reduced individual lifespan could have cascading demographic effects on population viability, although this remains to be explored.

In sum, populations evolving in contrasting environments showed divergent levels of developmental plasticity and associated oxidative stress and telomere length variation, despite the slight neutral genetic differentiation previously described. These results emphasize the importance of including physiological measurements in the study of phenotypic plasticity, in order to be able to understand the underlying mechanisms of particular evolutionary and ecological processes.

Ethics. This study was approved by the Animal Bioethics Committee from Doñana Biological Station (#12-53_Gomez) and all procedures performed involving the animals were in accordance with the ethical standards of the department and were approved by the Swedish Board of Agriculture (C21/14).

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Additional File 1

I.

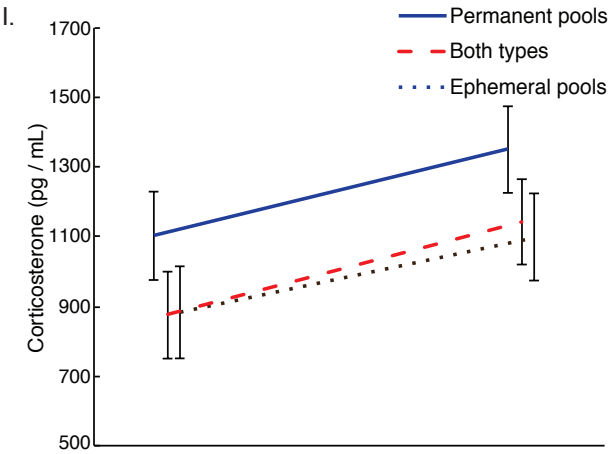

Figure I. The effect of pool drying on corticosterone levels (in pg/mL) in *Rana temporaria* tadpoles at 42 Gosner stage from three island habitats: islands with only permanent pools (blue line), islands with a mixture of ephemeral and permanent pools (dashed red line), and islands with only temporary pools (dotted black line). Data are least square means ± standard error.

II.

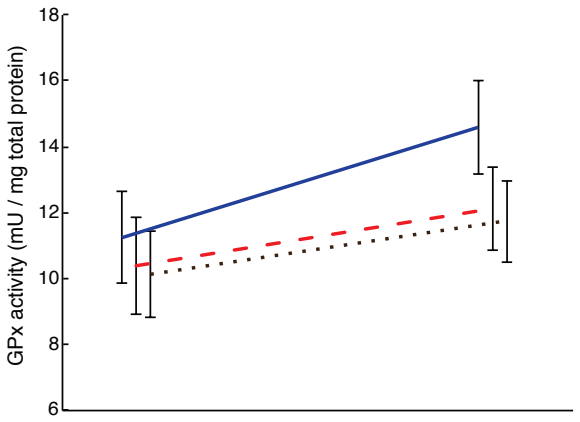

Figure II. The effect of pool drying on glutathione peroxidase activity (GPx, in mU/mg og total protein) in *Rana temporaria* tadpoles at 42 Gosner stage from three island habitats: islands with only permanent pools (blue line), islands with a mixture of ephemeral and permanent pools (dashed red line), and islands with only temporary pools (dotted black line). Data are least square means ± standard error.

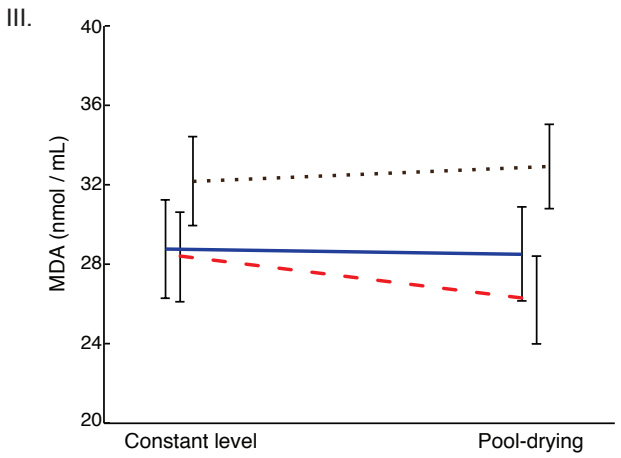

Figure III. The effect of pool drying on malondialdehyde concentration (MDA, in nmol MDA/mL) in *Rana temporaria* tadpoles at 42 Gosner stage from three island habitats: islands with only permanent pools (blue line), islands with a mixture of ephemeral and permanent pools (dashed red line), and islands with only temporary pools (dotted black line). Data are least square means ± standard error.

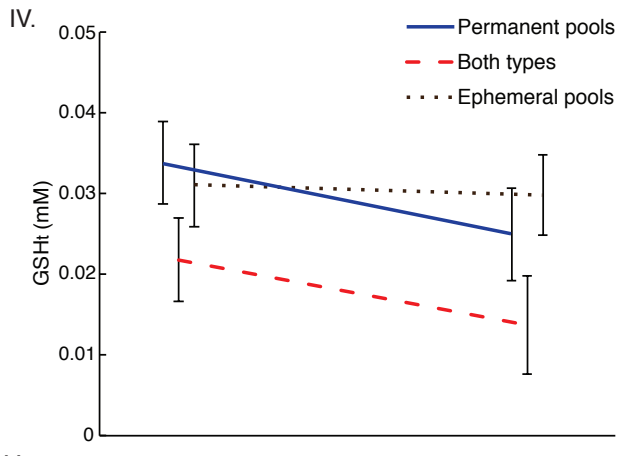

Figure IV. The effect of pool drying on total reduced glutathione (GSHt, in mM) in *Rana temporaria* tadpoles at 42 Gosner stage from three island habitats: islands with only permanent pools (blue line), islands with a mixture of ephemeral and permanent pools (dashed red line), and islands with only temporary pools (dotted black line). Data are least square means ± standard error.

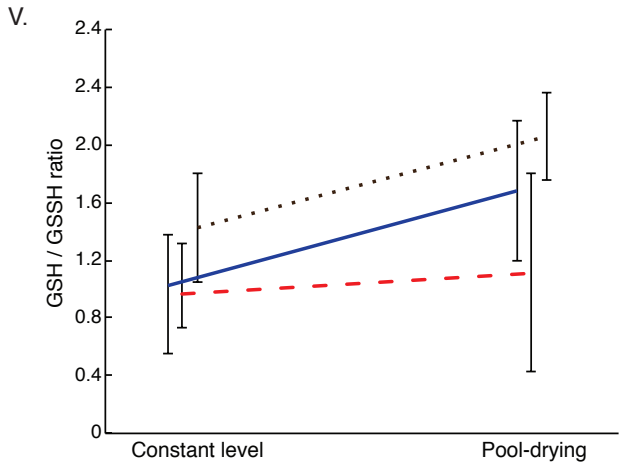

Figure V. The effect of pool drying on reduced/oxidized glutathione ratio (GSH/GSSG ratio) in *Rana temporaria* tadpoles at 42 Gosner stage from three island habitats: islands with only permanent pools (blue line), islands with a mixture of ephemeral and permanent pools (dashed red line), and islands with only temporary pools (dotted black line). Data are least square means ± standard error.

APPENDIX I

Comparing techniques for measuring corticosterone in tadpoles

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Abstract

Glucocorticoids play a key role in mediating stress responses in vertebrates. Corticosterone (CORT) is the main glucocorticoid produced in amphibians, birds, and reptiles, and regulates several metabolic functions. The most common methods for quantifying CORT are competitive binding immunoassays: radioimmunoassay (RIA) and enzyme immunoassay (EIA). RIA has been broadly used since the 1980's but it requires radioactivity. Commercial EIA kits permit quantifying hormone levels without radioactivity although the requirement for a larger sample volume may be a strong limitation for measurements involving larval amphibians. Here we used *Xenopus laevis* tadpoles to compare the performance of three commonly used procedures for determination of CORT: RIA on a chloroform extract of whole-body homogenate, EIA on plasma, and EIA on supernatant of whole-body homogenate. We treated tadpoles with exogenous CORT at 0, 25, 50, and 100 nM. RIA could distinguish between 0 and 25 nM, and EIA on plasma between 0 and 50 nM, whereas whole-body homogenate EIA only detected significant differences between 0 and 100 nM. Each procedure presents advantages and disadvantages regarding sensitivity, the use of radioactivity, sample size, handling time, and economic cost. RIA is preferred when studying small-bodied animals from which blood samples cannot be

obtained. When CORT level differences are intermediate and blood sampling is possible, EIA on plasma is a good non-radioactive alternative. EIA on whole-body homogenates may be useful to assess qualitative changes in CORT levels when considerable differences are expected. Finally, we discuss our findings in the context of previous studies on CORT in amphibians.

Keywords: Amphibians; Corticosterone; Enzyme immunoassay; Glucocorticoids; Radioimmunoassay; Stress

Introduction

Organisms make physiological adjustments in response to environmental fluctuations, whether natural or anthropogenic, through hormonal regulation (Johnson et al. 1992; Nussey and Whitehead, 2001; Becker et al. 2002). In vertebrates, the main hormonal response to environmental perturbations is based on the activation of the hypothalamic-pituitary-adrenal (HPA) axis to release corticotrophin-releasing hormone (CRH) (Miller and O'Callaghan, 2002; Denver, 2009). CRH stimulates the anterior pituitary to secrete the adrenocorticotropin hormone (ACTH) (Raffin-Sanson et al. 2003) followed by release of glucocorticoids (GCs) from the adrenal gland (Aguilera, 1994). Although the activation of this endocrine pathway is rapid, the GC level increase varies among species and individuals and can take around 3-5 min in vertebrates (Cash et al. 1997; Sockman and Schwabl, 2001; Romero and Romero, 2002). Corticosterone (CORT) is the main GC involved in the stress response in reptiles, birds, and amphibians whereas cortisol is the main one in fish and mammals (Sapolsky et al. 2000; Romero, 2004; Denver, 2009). Moreover, GC levels are associated with health condition, developmental rate, metabolism, and immune function (Denver et al. 2002; Walker et al. 2005; Wikelski and Cooke, 2006; DuRant et al. 2008; Davis et al. 2008), and they are commonly studied in developmental, ecological, and conservation studies (Romero, 2004; Busch and Hayward, 2009).

The most common methods for quantifying hormones are competitive binding immunoassays: radioimmunoassay (RIA) and enzyme immunoassay (EIA) (Sherrif, 2011; Narayan, 2013). Other techniques such as gas or liquid chromatography coupled with mass spectrometry have been successfully, though less frequently, used (Webb et al. 2007). RIA has been used extensively for over three decades and is a very sensitive technique to determine the concentration of antigens (e.g. hormones). RIA, however, requires the use of radioactive isotopes, specialized equipment, and a laboratory classified for handling radioactive materials. Moreover, radioactivity is increasingly avoided in laboratories and institutions because of the health risk and paperwork it entails.

In the 1970's, the development of commercial EIA kits allowed hormonal assays without the use of radioactivity. Commercial EIA kits are commonly designed for plasma or urine samples, providing information on circulating hormones (Wada et al. 2007; Mills et al. 2010) at competitive prices. However, when studies involve small species or life stages (e.g. embryos or larvae from amphibians, reptiles, and fish), obtaining the minimum required amount of plasma or urine to conduct EIA may prove challenging. Consequently, some researchers are attempting to conduct EIA using commercial kits on supernatant of whole-body homogenate instead of plasma (McMahon et al. 2011; Burraco et al. 2013). Clearly this would yield a diluted hormone sample compared to blood, and intracellular hormone may be included in the measurement. It is unclear whether this would yield comparable data to the other procedures or has a similar sensitivity to detect differences among study groups (e.g. species, populations, experimental treatments). Reduced specimen size may render whole-body supernatant EIA the only viable alternative to RIA, unless plasma from a large number of individuals could be pooled

together. However, losing the possibility to study CORT variation at the individual level may be undesirable because increasing the minimum required number of experimental individuals raises practical and bioethical issues. The choice of hormonal assay is therefore subject to various considerations depending on the study system, experimental design, infrastructure, and budget availability.

Amphibians are ideal to compare different procedures for determining GC levels as they have been used as a model group to analyze the role of GCs in multiple physiological processes, especially regarding the timing of metamorphosis (Denver et al. 2002; Gomez-Mestre et al. 2013). However, determining CORT levels in larval amphibians often poses difficulties derived from the small size of individuals of some species.

Here we compare the performance of three procedures for measuring CORT using tadpoles of the African clawed frog (*Xenopus laevis*): RIA on chloroform extract of whole-body, EIA on plasma, and EIA on supernatant of whole-body homogenate. We compare CORT estimates obtained from tadpoles treated with different amounts of exogenous CORT and discuss the suitability of each procedure with respect to sensitivity and discrimination power, but also to bioethical, logistic, and budgetary criteria. We also review and discuss the methodology used for CORT determination in anurans over the last 20 years.

Material and Methods

Experimental setup

We collected egg clutches from three *Xenopus laevis* pairs from a breeding colony at Centro Andaluz de Biología del Desarrollo (Seville,

Spain). After hatching, 240 tadpoles were reared for 60 days in 2.7 L buckets (5 tadpoles/bucket) until they reached Nieuwkoop and Faber developmental stages 52-54 (Nieuwkoop and Faber, 1994). At this stage range, tadpoles show fore and hind limbs in paddle stage and the hind limbs without feet and longer than broad (mean length 55 mm, Nieuwkoop and Faber, 1994). During this rearing period, water was renewed twice a week and tadpoles were fed 30 mg of ground rabbit chow every other day. The experimental units were distributed across shelves in a walk-in chamber set at constant 20 ºC, and a 12:12 light-dark cycle.

When the tadpoles reached NF 52-54 we treated them with exogenous CORT (0 nM, 25 nM, 50 nM, or 100 nM). Each CORT treatment was replicated 12 times for a total of 48 experimental units. Water was changed daily, and at each water change tadpoles were fed 30 mg of ground rabbit chow and CORT (C2505, SIGMA) was added. Tadpoles have permeable skin (Bentley, 1971; Parson, 1994), so CORT diluted in water is directly uptaken via skin absorption. To reach the experimental concentrations, we prepared stock solutions of CORT diluted in absolute ethanol so that an addition of 200 μL of any of them to 2.7 L of water in the experimental containers would result in the target concentrations of 25, 50, and 100 nM. We first prepared a 1350 μM stock solution (23.5 mg of CORT in 50 mL of ethanol) from which the 100 nM experimental concentration would be obtained, and then prepared two stocks at lower concentrations by serial 1:1 dilutions with absolute ethanol. Buckets in the control (no CORT) received 200 μL of ethanol each time. The stock concentrations are based on previous studies (Glennemeier and Denver, 2002) and were kept at -80 °C. Three days after applying the hormonal treatments we collected tadpoles from each bucket for CORT measurements, randomly assigning them to RIA (one tadpole

per bucket), EIA on plasma (three tadpoles pooled per bucket) and EIA on whole-body homogenate (one tadpole per bucket). We did not find differences in weight among treatments at the end of the experiment, and tadpoles weighed an average of 223 ± 9 mg (SE).

Plasma samples for EIA were obtained through heart puncture. Tadpoles deeply anesthetized with MS-222 (Ethyl 3-aminobenzoate methanesulfonate, Sigma), were placed under a dissecting scope, and blood was extracted via cardiac puncture with a non-heparinized insulin syringe (BD Micro-Fine Insuline U-100 0.5 ml). Blood from all three individuals (ca. 20 μL per tadpole) from each bucket was pooled in heparinized tubes and then centrifuged at 4000 rpm, at 4 °C for 20 minutes to obtain plasma (Gomez-Mestre et al. 2013). Plasma samples were stored at -80 °C in 0.5 ml eppendorf tubes until assayed. The average handling time per individual for the blood extraction procedure from immersion in anesthetic to complete collection of the blood sample was 193 s ± 7.5 SE.

Individuals assigned to RIA were dipnetted from their containers, euthanized with MS-222 (Ethyl 3-aminobenzoate methanesulfonate), dissected to remove the gut, snap-frozen in liquid nitrogen, and preserved at -80 ºC until used. For whole-body homogenate EIA, tadpoles were euthanized with MS-222, dissected to remove the gut, ground up with a homogenizer (MICCRA D-1), and centrifuged at 4000 rpm at 4 ºC for 15 min. The resulting supernatant was preserved at -80 ºC until used (Burraco et al. 2013).

Radioimmunoassay

Specimen samples were homogenized (except the gut) as described above centrifuging and extracting the hormone from the

supernatant with chloroform and ion-exchange chromatography prior to RIA following previous protocols (Denver, 1993). Lower detection limit was 0.20 pg/mL/mg.

Plasma and whole-body homogenate EIAs

We took 50 μL of either plasma or homogenate (supernatant) from each sample and conducted EIA with a commercial kit following manufacturer specifications (Cayman Chemical Company – catalog nº 500655). The CORT determination with this EIA kit is based on the competition between a CORT-acetylcholinesterase conjugate and CORT, for a limited number of CORT-specific sheep antiserum binding sites which bind to the rabbit polyclonal anti-sheep IgG that previously was attached to the well. For this, each plate was incubated for two hours at room temperature on an orbital shaker. Ellman's reagent, which contains a substrate for acetylcholinesterase, was added to the wells to develop the assay. After incubating for 60-90 minutes to achieve a stable end point in an orbital shaker in the dark, absorbance was read at a wavelength of 412 nm, and CORT concentration was determined based on standard curves run in duplicate on each plate. According to the manufacturer, the detection limit (80 % B/B_0) is approximately 40 pg/mL, and cross reactivity with other tested steroids is below 1%. It was not the aim of this study to compare the efficacy of the various commercial kits available, and differences among kits from different suppliers are likely to affect the outcome due to differences in their antibody specificity or detection limits. Moreover, most of these kits are optimized for use with human samples, and in our experience not all work well with amphibian samples.

Statistical tests

We assayed 38 tadpoles through RIA, with 9-10 replicates per treatment. For EIA on plasma, we extracted blood from 135 tadpoles. We obtained the required 50 μL pooling plasma from three tadpoles per bucket, yielding 6-7 replicates per treatment. For some pooled samples, the total volume obtained was insufficient to run the assay. In whole-body homogenate EIA we obtained 9-11 replicates per treatment and we used 40 tadpoles in total. All statistical analyses were conducted in R version 2.14-1 (R Development Core Team 2007). We checked the parametric assumptions testing normality of the data via analysis of residuals distribution and homoscedasticity using Barlett's tests (bartlett.test). Data from RIA and whole-body homogenate EIA were heteroscedastic and not normally distributed, and hence we fitted generalized linear models with a negative binomial distribution (function glm.nb in MASS package, version 7.3-22) to test for differences among CORT treatments. We then ran *post-hoc* tests using the glht function (multcomp package, version 1.2-13). Plasma EIA data, however, met parametric assumptions and therefore we fitted instead a general linear model (aov function) followed by *post-hoc* Tukey tests. We tested the effect of handling time including it as a covariate in the model. We calculated intraclass correlations to determine the reliability and consistency of the measurements within treatments for each procedure with the ICC function (ICC package, version 2.2.1).

Results

Handling time for individuals preserved for whole-body homogenate (RIA or EIA) was limited to ~30 s in the anesthetic (MS-222). Handling time during blood sample collection had no significant effect on CORT levels (coefficient of regression: 0.19, $P = 0.355$, $R^2 = 0.037$).

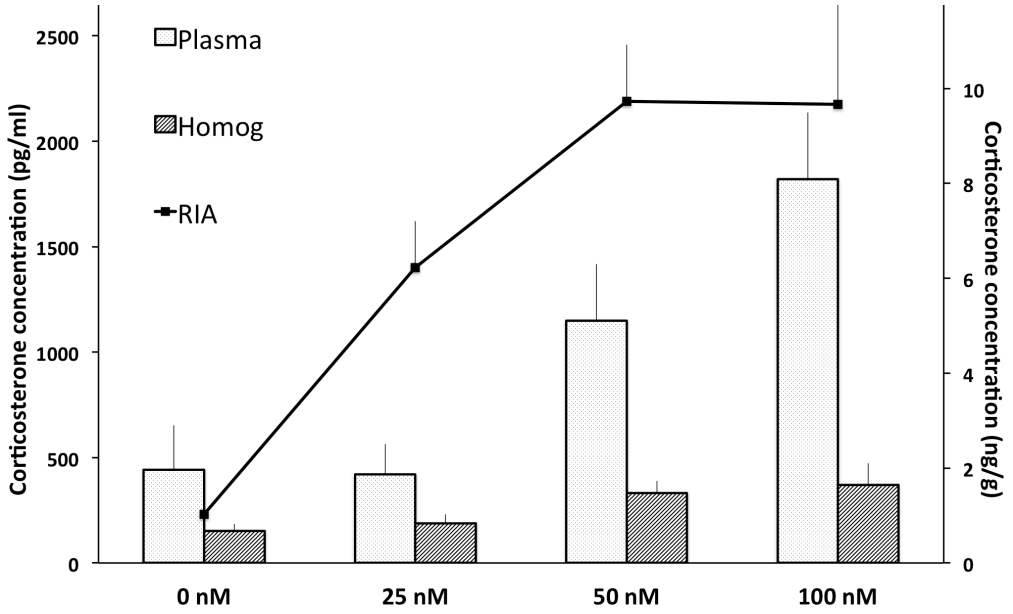

Figure 1. Corticosterone concentration measured applying EIA plasma (light grey bars, left Y axis), EIA whole-body homogenate (dark grey bars, left Y axis), or RIA (black line, right Y axis). Error bars indicate standard error of the mean.

We detected significant changes among treatments in CORT concentration, regardless of the procedure used (Figure 1; overall tests: $\chi^2 = 50.605$, df = 3, $P < 0.001$ for RIA; $F_{3,22} = 10.08$, $P < 0.001$ for EIA on plasma; $\chi^2 = 7.89$, df = 3, $P = 0.017$ for EIA on whole-body). For RIA data, *post-hoc* tests (Table 1) showed significant differences among the control treatment and each exogenous CORT treatment (25, 50 and 100 nM; all $P < 0.001$). The CORT levels increased on average by 6.1-fold between 0 nM and 25 nM treatments, and by up to 9.52 and 9.47-fold in the 50 and 100 nM treatments in comparison to the control treatment. Plasma EIA showed that CORT increased

4.12 times in the 100 nM treatment with respect to the control (P = 0.001). We also found significant differences between 25 nM and 50 nM (P = 0.045), and 25 nM and 100 nM (P < 0.001), but not between the control and 25 mM. We found noticeable changes in the CORT levels between 0 nM-50 nM and 50 nM-100 nM but they were marginally not significant (Table 1). Overall EIA on plasma showed the same number of significant pairwise differences among treatments that RIA did, and in both cases we found significant differences between treatments that differed in 25 nM of CORT. However, RIA detected significant differences between the lowest concentrations of corticosterone (0 nM-25 nM) and EIA on plasma detected better than RIA differences when higher concentrations were added (50 or 100 nM). The model fit on whole-body homogenate EIA indicated a significant effect of hormonal treatments. *Post-hoc* tests only found significant differences between 0 nM and 100 nM treatments (P = 0.002) although differences between 0 nM-50 nM and 25nM-100 nM neared statistical significance (P ~ 0.07).

Comparing the absolute values (in pg/ml) obtained in plasma EIA with those estimated from whole-body homogenate EIA (Figure 1) we found diluted levels in the homogenate, yielding values in the range of 2.1 to 4.6 times lower concentrations than in plasma. This comparison is not possible with the RIA values because these are given per mass unit (pg/ml/mg).

The intraclass correlation coefficient (ICC) provides an idea of the consistency of the data within experimental treatments. ICCs indicated a higher consistency of data from EIA on plasma (ICC = 0.56) and RIA (ICC = 0.40) than EIA on supernatant of whole-body homogenate (ICC = 0.14).

Table 1. *Post-hoc* Tukey tests for differences among treatments in RIA, EIA on plasma, and EIA on whole-body homogenates.

Post-hoc **(*P*-adjusted)**	0-25 (nM)	0-50 (nM)	0-100 (nM)	25-50 (nM)	25-100 (nM)	50-100 (nM)
RIA	< 0.001	< 0.001	< 0.001	0.093	0.093	0.980
EIA (plasma)	0.7497	0.0627	0.0011	0.0454	< 0.001	0.0615
EIA (homog)	0.475	0.071	0.023	0.229	0.071	0.4988

Discussion

The comparison of RIA on whole-body extract, EIA on plasma, or EIA on supernatant of whole-body homogenate, revealed that RIA was a more sensitive procedure for detecting low CORT values than the EIA procedures. RIA on whole-body extract was the only procedure capable of detecting differences between control tadpoles and tadpoles treated with 25 nM CORT (Table1). RIA, however, was rapidly saturated and could not distinguish tadpoles treated with more than 50 nM. Diluting the samples prior to the assay, however, could solve this problem of RIA saturation. EIA on plasma samples was less sensitive than RIA at low CORT levels but also allowed detection of differences between treatments that differed by 25 nM (i.e. 25nM *vs* 50 nM). Moreover, EIA showed no indication of saturation in our study, indicating a larger dynamic range for detecting significant differences. This characteristic of EIA on plasma may be advantageous when species have unknown basal CORT levels and limited sample volumes preclude use of dilution series to assess assay saturation.

EIA on whole-body homogenate supernatant showed the lowest sensitivity since we could only detect significant differences between tadpoles treated with 100 nM CORT and those with no CORT added. Nevertheless, EIA on whole-body homogenate supernatant showed the same trend as EIA on plasma (Fig. 1) but with rather dampened values, most likely a consequence of dilution with CORT-free body fluids. The most consistent procedure (i.e. the one with higher intra-class correlation coefficient) was EIA on plasma, followed by RIA.

CORT concentrations measured in previous studies in natural or semi-natural conditions reported values that were within the range in which the three procedures can detect significant differences. There is much information about CORT levels obtained via RIA on whole-body extracts. For example, Glennemeier and Denver (2002) obtained values between 0.4 and 0.8 ng/g in *Rana pipiens* differentially treated with exogenous CORT; Maher et al. (2013) measured CORT in *R. sylvatica* tadpoles and juveniles differentially exposed to predator presence and obtained values between 0.5 and 4 ng/g. Likewise, Belden et al. (2010) measured CORT in *R, sylvatica* tadpoles repeatedly to estimate capture stress in natural ponds (0.2-0.5 ng/g), and in *Scaphiopus holbrooki* raised in 1000 L mesocosms (less than 0.1 ng/g). Regarding CORT values measured with EIA on plasma, pre- and postmetamorphic *Pelobates cultripes* individuals exposed to pond drying reported CORT concentrations between 0.1-0.4 ng/mL (Gomez-Mestre et al. 2013). Burraco et al. (2013) measured CORT on supernatant of whole-body homogenate and values were around 0.1 and 0.2 ng/mL in tadpoles exposed to predators and herbicide.

The study of stress responses in amphibians in the last decades has required conducting corticosterone assays on several species.

We found 56 studies that determined CORT in anurans in the last two decades (Table 2). Some of these studies included several species or different life stages, which we considered as separate corticosterone measurements (N = 70). The majority of such measurements were obtained using RIA (N = 46). Within these RIA studies, we found 11 instances where CORT was measured on plasma: six in adults, four in juveniles, and only one in tadpoles (which included one or two tadpoles per sample; Wright et al. 2003). This bias towards adult and juvenile stages in the analysis of plasma samples likely reflects the difficulty in obtaining blood samples in tadpoles due to its reduced size. Consequently, most tadpole studies opted for whole-body chloroform extracts (N = 31, Table 2).

Table 2. Overview of corticosterone studies in anurans from 1993 to 2013. We have only included studies that used RIA or EIA to determine the corticosterone levels. Some studies have multiple entries because they included several analysis, e.g. with different species or individual' stages. The full references are included in the "Suplementary material".

Techniques	Body parts	Stages	Species	References	Year
RIA					
	plasma	adult	*B. terrestris*	Hopkins et al.	1997
		adult	*B. terrestris*	Hopkins et al.	1998
		adult	*B. terrestris*	Ward and Mendonça	2005
		adult	*R. catesbeiana*	Wright et al.	2003
		adult	*R. sylvatica*	Crespi and Warne	2013
		adult	*X. laevis*	Hayes et al.	2006
		juvenile	*S. hammondii*	Crespi and Denver	2005
		juvenile	*X. laevis*	Kloas et al.	1997
		juvenile	*X. laevis*	Crespi et al.	2004
		juvenile	*X. laevis*	Hu et al.	2008

	tadpole (1-2 per sample)	*R. catesbeiana*	Wright et al.	2003
whole-body	juvenile	*R. sylvatica*	Belden et al.	2007
	juvenile	*R. pipiens*	Glennemeier and Denver	2002
	juvenile	*R. sphenocephala*	Peterson et al.	2009
	juvenile	*X. laevis*	Glennemeier and Denver	2002
	tadpole	*B. boreas*	Hayes	1997
	tadpole	*B. boreas*	Hayes and Wu	1995
	tadpole	*H. regilla*	Belden et al.	2005
	tadpole	*H. versicolor*	Chambers	2011
	tadpole	*R. cascadae*	Belden et al.	2003
	tadpole	*R. clamitans*	Fraker et al.	2009
	tadpole	*R. pipiens*	Glennemeier and Denver	2002
	tadpole	*R. pipiens*	Glennemeier and Denver	2002
	tadpole	*R. pipiens*	Glennemeier and Denver,	2001
	tadpole	*R. pipiens*	Glennemeier and Denver	2002
	tadpole	*R. sphenocephala*	Peterson et al.	2009
	tadpole	*R. sylvatica*	Belden et al.	2010
	tadpole	*R. sylvatica*	Fraker et al.	2009
	tadpole	*R. sylvatica*	Chambers	2011
	tadpole	*R. sylvatica*	Warne et al.	2011
	tadpole	*R. sylvatica*	Belden et al.	2007
	tadpole	*R. sylvatica*	Crespi and Warne	2013
	tadpole	*R. sylvatica*	Middlemis et al.	2013
	tadpole	*R. sylvatica*	Reeve et al.	2013
	tadpole	*R. temporaria*	Dahl et al.	2012
	tadpole	*S. bombifrons*	Ledón-Rettig et al.	2009
	tadpole	*S. hammondii*	Denver	1998
	tadpole	*S. hammondii*	Crespi and Denver	2004
	tadpole	*S. hammondii*	Crespi and Denver	2005

		tadpole	*S. hammondii*	Denver	1997
		tadpole	*Sc. couchii*	Ledon-Rettig et al.	2009
		tadpole	*Sc. couchii*	Ledon-Rettig et al.	2010
		tadpole	*X. laevis*	Kloas et al.	1997
		tadpole	*X. laevis*	Glennemeier and Denver	2002
		tadpole	*X. laevis*	Boorse and Denver	2004
		tadpole	*X. laevis*	Hu et al.	2008
EIA					
	plasma	adult	*R. marina*	Brown et al.	2011
		tadpole	*P. cultripes*	Gomez-Mestre et al.	2013
	whole-body	tadpole	*O. septentrionalis*	McMahon et al.	2011
		tadpole	*P. cultripes*	Burraco et al.	2013
	urinary CORT	adult	*L. wilcoxii*	Kindermann et al.	2012
		adult	*L. wilcoxii*	Kindermann et al.	2013
		adult	*M. Fasciolatus*	Graham et al.	2013
		adult	*P. vitiana*	Narayan et al.	2010
		adult	*P. vitiana*	Narayan et al.	2010
		adult	*P. vitiana*	Narayan and Hero	2011
		adult	*P. vitiana*	Narayan et al.	2012
		adult	*P. vitiana*	Narayan et al.	2013
		adult	*P. vitiana*	Narayan et al.	2013
		adult	*P. vitiana*	Narayan et al.	2013
		adult	*R. marina*	Narayan et al.	2011
		adult	*R. marina*	Narayan et al.	2012
		adult	*R. marina*	Narayan et al.	2012
		adult	*R. marina*	Narayan et al.	2012
		adult	*R. marina*	Narayan et al.	2012
		adult	*R. marina*	Narayan et al.	2012
		adult	*R. marina*	Narayan et al.	2013

	adult	*R. marina*	Narayan et al.	2013
in water pond	tadpole	*A. obstetricans*	Gabor et al.	2013
	tadpole	*A. muletensis*	Gabor et al.	2013

Studies with EIA used different organs or fluids or else incorporated various modifications of the technique. For example, we found 18 instances where CORT levels were assayed in urine of adult amphibians and just one where CORT was determined in plasma. Studies on tadpoles applied EIA in various ways. Two studies ran assays on whole-body homogenates: one of them first extracted the hormone with tritiated CORT as a tracer (McMahon et al. 2011), and the other study determined CORT levels from pond water (Gabor et al. 2013).

RIA has thus been the preferred technique for quantifying CORT in anurans in the last two decades, most often applied to extracts from whole-body tadpole homogenates. EIA was conducted on various sample types, and was preferred when assaying urine or blood samples. Various factors need to be taken into account in order to decide which method to use (Table 3). RIA is widely used but requires radioactivity, which is not implemented in many research institutions. Regarding budgetary aspects the use of EIA can be favored over the use of RIA, as EIA commercial kits are available at competitive prices that do not require additional disposal costs as with radioactive materials generated by RIA. Our results indicate that EIA on plasma samples is a reasonable non-radioactive alternative to RIA, with two caveats. First, EIA has lower sensitivity at low CORT levels. Second, many studies will focus on study systems where individuals are too small for collecting sufficient plasma samples for commercial kits (typically requiring ca. 50 µL), especially so in early ontogenetic stages. Thus, obtaining sufficient plasma samples from small organ-

isms may require pooling blood from several individuals into a single sample, hence requiring the sacrifice of many more individuals than are otherwise required for either RIA or EIA on whole-body homogenate. In our study each plasma sample for EIA required pooling blood from three *X. laevis* tadpoles whereas single tadpoles were allocated to either RIA or EIA on whole-body supernatant. This of course raises conservation and bioethical concerns, especially when dealing with threatened or vulnerable species, which in the case of amphibians includes ca. 43 % of all known species (Hoffmann et al. 2010; LesBarrères et al. 2014). Moreover, an increase in the number of tadpoles in the study can pose some logistical problems in laboratories as well as during field campaigns. Thus, studies will have to either increase the total number of replicates to account for the need to pool samples, or increase the number of tadpoles per experimental unit. Besides, rearing more tadpoles per unit requires larger containers since larval density in itself directly results in increased CORT levels (Hayes, 1997).

Another potential confounding factor in determining CORT levels is the effect of handling time. GCs levels can increase inordinately after only 3-5 minutes from the first contact with the individual (Wingfield et al. 1982; Romero and Romero, 2002; Cash et al. 1997) and usually reach peak levels after 15-30 min after exposure to the stressor (De Kloet et al. 2005). Handling times are not a concern for procedures like RIA and whole-body homogenate EIA in which animals can be quickly (under 3-5 min) euthanized and preserved (e.g. through immersion in MS-222 followed by snap freezing of the whole body). Obtaining blood samples from tadpoles, however, is a more laborious procedure and some training is required to reduce handling times under 3-5 min. Therefore, handling times may become a

likely source of error and need to be accounted for in the analyses. For this reason, RIA and whole-body homogenate EIA are the best method to measure GC in field studies. EIA on supernatant of whole-body homogenates has the advantages of not requiring radioactivity, avoiding potential error from increased handling times, and requires fewer individuals than plasma EIA. Conversely, it constitutes a more conservative test of differences among test groups (experimental treatments, populations, species) (Burraco et al. 2013), as it is less sensitive than either RIA or plasma EIA.

Table 3. Main characteristics of EIA in plasma, EIA in homogenate and RIA. + symbols indicate the viability of each procedure.

	Ind/sample (in *X. laevis* tadpoles)	Freeze and measured?	USD / sample	Radioactivity	Use in field studies?	Use in small individuals	ICC-value
RIA	1	YES	3 *	YES	+++	++	++
EIA on plasma	3 (at least)	NO	2.8	NO	+	+	+++
EIA on homogenate	1	YES	2.8	NO	+++	+++	+

*plus radioactive disposal costs

The choice of procedure for each study should thus be made considering methodological limitations and tractability for each study system. RIA is still the golden standard in eco-physiological studies because it is most sensitive, it is appropriate for small larvae, and can be used to compare CORT levels using different tissues or on whole-body homogenates. However, plasma EIA is a good non-radioactive alternative that has been used to obtain significant results in response to natural perturbations such as pond drying (Gomez-Mestre et al.

2013). EIA on whole-body homogenate supernatant could detect qualitative differences in CORT levels among experimental groups, and may be of use if individuals are small and radioactive assays cannot be implemented.

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APPENDIX II

Validated flow cytometry allows rapid quantitative assessment of immune responses in amphibians

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Abstract

Assessments of amphibian immune state have been commonly made through indirect methods like phytohemagglutinin (PHA) injections, or by direct methods like cell counts from blood smears. Here we validate a simple method to assess immune responses in amphibians by means of flow cytometry with a fluorescent lipophilic dye (3,3' Dipentyloxacarbocyanine), which removes the need for specific antibodies. We experimentally altered the immunological state of *Pelobates cultripes* tadpoles by exposing some to exogenous corticosterone. We then determined the immune state of each tadpole through both blood smears and flow cytometry. We found that both techniques showed similar patterns of the proportion of white blood cells. Once validated, flow cytometry also allowed quantitation of changes in absolute number of white cells. We discuss the suitability of both techniques attending to the accuracy of each technique, body size requirements, or the tractability in field studies.

Keywords: Amphibians; Blood smears; Flow cytometry; Immune system; Leukocyte count

Introduction

The study of organisms' immune state is essential for understanding physiological responses against environmental challenges and their likely trade-off with immunological defenses. In vertebrates, stress responses are mainly regulated via activation of neuroendocrine pathways like the hypothalamic-pituitary-adrenal (HPA) axis (Denver, 2009). Such stress responses usually involve glucocorticoids secretion, which result in alterations of the proportion of white blood cells (leukocytes) (Davis et al. 2008). Leukocytes comprise three cell types: lymphocytes, granulocytes (subcategorized as neutrophils, eosinophils, and basophils), and monocytes. Stress-induced glucocorticoid secretion cause elevations of the granulocytes:lymphocytes (G:L) ratio, particularly in increased neutrophils:lymphocytes (N:L) ratio (Davis et al. 2008). Elevated glucocorticoid levels can also produce lymphopenia, i.e. decreased number of lymphocytes in the blood. It can also cause granulocytosis or neutrophilia, i.e. increased number of granulocytes or neutrophils in the blood (Davis et al. 2008). Consequently, the G:L ratio has been described as a reliable biomarker for chronic stress (Goessling et al. 2015)

Assessments of the immune status and immune responses in amphibians have typically been carried out by either indirect methods such as phytohaemagglutinin (PHA) injection or by direct methods

such as cell counts from blood smears, even though flow cytometry has been successfully implemented in taxa such as rodents, birds or reptiles (Demas et al. 2011). PHA, a lectin found in plants, induces a localized inflammation around the injection site followed by an influx of neutrophils, eosinophils, macrophages, and T-lymphocytes between 4-12 h after the injection (Brown et al. 2011). The use of PHA injection provides an indirect measurement of the immune state of the individual since it causes local swelling and the extent of such swelling is indicative of the measure of T-cell dependent immunocompetence (Brown et al. 2011). PHA injection is a suitable technique for use in non-model organisms, and it provides a quick and simple assessment of the immune state of the organisms. However, this method may be intractable, for small-bodied species, and requires repeated sampling at specific time points which can make it difficult to perform in field studies (Demas et al. 2011). Moreover, it does not provide information regarding the nature of the immunological response in terms of the cell types involved. Direct cell counts from blood smears under the microscope have been the preferred approach for quantitation of immune responses in amphibians (Allender et al. 2008). In this technique blood is extracted and smeared over a glass slide and then stained with one or several dyes, allowing the identification of various types of white blood cells. May-Grünwald-Giemsa is one of the more broadly used blood staining procedures, and contains methylene blue (basic dye), related azures (also basics), and eosin (acidic dye). The combination of dyes stains granules, cytoplasm and nuclei of the different cell types in a range from purple to red. However, leukocyte count through blood smears is laborious, requires a fair amount of training to distinguish cell types, and requires a considerable amount of blood to be extracted per individual (ca. 15-20 µL per smear).

This could pose an important methodological limitation in species with hypovolemia like amphibians, and even more so if blood is to be extracted from larvae.

The implementation of flow cytometry in the last decades has greatly improved, the study of the immune response in several taxa, as current cytometers are increasingly affordable, smaller, and easy to use. Flow cytometry is linked to the use of fluorescent dyes that absorb light at a certain wavelength and bind specifically to particular cellular components, providing a rapid, qualitative, and quantitative analysis of white blood cells (Brown and Wittwer, 2000). The fundamentals of flow cytometry are based on physical characteristics of the cells such as size and complexity, which are represented by forward angle light scatter (FSC) and by right-angle light scatter (SSC), respectively. The use of flow cytometry in immunology is commonly based on species-specific antibodies that specifically recognize leukocyte surface receptors. However, the development of antibodies is expensive and is typically constrained to model-organisms. Fortunately, the use of fluorescent lipophilic dyes such as 3,3' Dipentyloxacarbocyanine iodide –$DiOC_5$(3)- has removed the need for specific antibodies in the identification of blood cells via cytometry (Uchiyama et al. 2005). However, up until now, there had been no studies quantifying white blood cells through flow cytometry in amphibians other than studies on *Xenopus laevis* for which specific antigens had been developed to recognize specific leukocytes (Colombo et al. 2015). Therefore, flow cytometry with non-specific fluorescent dyes could be most valuable for immunological quantification in amphibians. This technique only requires ca. 1 µL of blood, which is a major improvement with respect to blood smears in amphibian studies, especially if working with larvae. Nevertheless, implementation of flow cytometry requires validation of the output in order to delimit the gates associated with each cell type, and that is our aim in this study.

Here we validate the use of flow cytometry with a non-specific lipophilic dye to determine immune response in tadpoles of the western spadefoot toad (*Pelobates cultripes*), by comparing its results with those obtained through direct counts from blood smears. Moreover, we determined the ability of both techniques to detect changes in immune state induced by a simulated stress response. We experimentally manipulated the immune state of tadpoles by adding exogenous corticosterone (CORT), the main glucocorticoid involved in stress response in amphibians (Denver, 2009). We then quantified the causal relationship between CORT levels and changes in the proportion of the different leukocyte types.

Material and methods

Sampling and experimental setup

In February 2014, we collected a small portion of one *Pelobates cultripes* clutch from the Biological Reserve of Doñana National Park (Huelva, Spain). The embryos were maintained in a wide tray filled with carbon-filtered dechlorinated tap water in a climate chamber set at 18 ºC and 12:12 light:dark photoperiod until hatching. Upon hatching, tadpoles (N = 28) were raised individually in 3-L buckets in a climate chamber set at 24 ºC and 12:12 ligth:dark cycle. Tadpoles were fed rabbit chow *ad libitum*. The experiment started 60 days after hatching, when tadpoles were in Gosner stage 33-34 (Gosner, 1960). Experimental units were randomly assigned to each of two treatments: no exogenous CORT (control), or 100 nM of exogenous CORT (stress induction; Glennemeier and Denver 2002). Each treatment was replicated 14 times. In the stress induction treatment we added 200 µL from a stock previously prepared at 1,350 µM of CORT

(23.5 mg of CORT in 50 mL of ethanol). Control containers received 200 µL of ethanol without CORT. Water was renewed each day and CORT or plain ethanol were added immediately after each water change. The CORT stock was stored at -80 ºC until used. After five days of experiment we collected all tadpoles. Tadpoles were anesthetized with MS-222 prior to blood extraction and were weighed on an analytical balance (CP324S, Sartorius). Blood was obtained via cardiac puncture with a 29G syringe (BD Micro-Fine Insuline U-100 0.5 ml) and was used for leukocyte count through either blood smear or flow cytometry. Blood samples for cytometry were introduced in heparinized tubes and kept over ice (covered in tissue paper to prevent freezing) and assayed within 10 min of collection.

Blood smears

We performed two blood smears per individual. Blood was smeared on glass slides and the smears were allowed to dry at room temperature and then fixed through immersion in absolute methanol for 10 seconds. Fixed blood smears were then stained using the May-Grünwald – Giemsa procedure. We covered the whole-slide (ca. 3 mL) with May-Grünwald (Panreac) for 5 min and then added the same amount of distilled water for 1 min. The mix was then discarded and we covered the slide with diluted Giemsa (1:20, Giemsa:distilled water; Panreac) for 20 min. Finally, the slides were rinsed with tap water and allowed to dry at room temperature. The stained smears were mounted with DPX (EukittTM Mounting Medium). Leukocyte identification and quantification was performed under the microscope at 100X magnification (ZEISS Immersion microscope, model Zi) using Oil DC. We counted a total of 200 white blood cells per smear. Although we differentiated among different types of granulocytes

through blood smears, we pooled the three granulocyte types together in order to compare with cytometric data.

Flow cytometry

We determined leukocyte formulae through flow cytometry following the protocol developed by Uchiyama et al. (2005) for birds. We diluted 1 µL of blood in 1 mL of Hank's balanced salt solution and we mixed with 1 µL of $DiOC_5(3)$ (Sigma-Aldrich, St. Louis, MO) previously diluted 1:10 in absolute methanol. Hank's solution maintains pH and osmotic balance of the cells and provides water and essential inorganic ions for the cell. $DiOC_5(3)$ is a fluorescent lipophilic dye, photostable when incorporated onto cell membranes. The mixtures of blood, Hank's solution, and $DiOC_5(3)$ were vortexed and incubated at room temperature for 3 min before leukocyte determination. We used a Guava Easy Plus cytometer (Guava Technologies, Hayward, California, USA). We cleaned the cytometer capillaries every 20 measurements with absolute methanol to minimize noise in the form of accumulated debris. All samples were run in duplicate. A maximum of 25,000 events were recorded per sample. Four different gates were defined, corresponding to four cellular types: erythrocytes, lymphocytes, granulocytes, and monocytes. These gates were defined attending to cellular characteristics such as size and complexity (see below).

Flow cytometry gating

A gate in flow cytometry is an electronic window that defines a cell population. Therefore, a proper gating strategy is a critical step in flow cytometry and the resulting output usually requires a microscopic validation (Naeim et al. 2008). For optimizing the resulting output, we first

defined gates attending to the regions described by Uchiyama et al. (2005) for each cell type. Then, we optimized these gates applying a gating strategy based on FSC, SSC and green fluorescence dot plot combination (Roussel et al. 2010). This gating strategy allowed us to avoid abnormal cells and to define homogeneous cell groups. Gates were defined using free-drawn regions for lymphocytes and granulocytes, and rectangular region for monocytes (see Figure 1A and 1B). The use of a single fluorochrome prevented possible fluorescence interference among cell types. Finally, we compared the G:L ratio determined through flow cytometry and blood smears in order to validate the method. After gating, we also quantified the absolute number of white blood cells (i.e. per µL of blood) in order to determine possible lymphopenia or granulocytosis after CORT exposure.

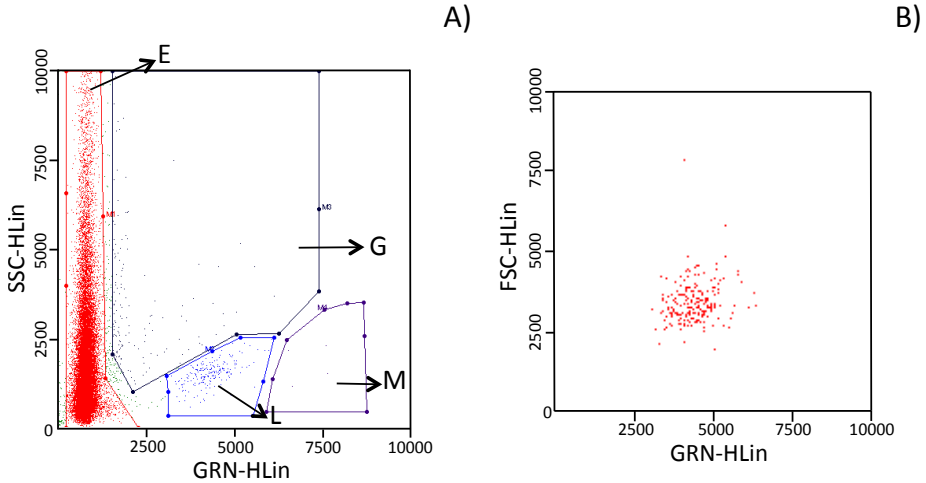

Figure 1. A) Gates of *Pelobates cultripes* blood cell populations revealed by their side scatter (SSC-HLin) and green fluorescence (GRN-HLin) properties. Erythrocytes, granulocytes, lymphocytes, and monocytes were designed as E, G, L, and M, respectively. In this example 24,073 erythrocytes, 202 lymphocytes, 146 granulocytes, and 4 monocytes were quantified. B) Example of a lymphocyte gate. Gates were optimized applying a gating strategy based on forward scatter (FSC-HLin) and green fluorescence (GRN-HLin) dot plot combination.

Statistical tests

All statistical analyses were conducted in R version 2.14.1 (R Development Core Team 2007). We fitted generalized linear models with a binomial error distribution to test for differences in leukocyte proportions. We estimated the granulocytes:lymphocytes ratio as Ln(granulocytes) – Ln(lymphocytes). To determine the consistency of our results we determined the coefficient of variation (CV %) within and among samples. We also estimated the reliability and consistency of the measurements within treatments by estimating the intraclass correlation coefficient using the ICC function (ICC package, version 2.2.1). We excluded monocyte counts from statistical analyses due to their low frequency in amphibian blood (Davis et al. 2008). The absolute count of lymphocytes and granulocytes was only estimated using data obtained via flow cytometry determination after validation of the method since the use of blood smears for this absolute leukocyte count can result inaccurate (Gering and Atkinson, 2004).

Results

Survival was 100 % throughout the experiment. Tadpoles exposed to exogenous CORT reduced their body mass by an average of 21.31 % ($F_{1,26}$ = 19.68; $P < 0.001$) compared to control tadpoles. We detected a significant increase of the G:L ratio in response to exogenous CORT addition, whether using blood smears or flow cytometry. The G:L ratio increased after CORT exposure by 71.42 % as estimated through flow cytometry ($F_{1,26}$ = 39.79; $P < 0.001$; Figure 2A), and by 136.69 % as estimated via blood smears ($F_{1,26}$ = 32.22 ; $P < 0.001$; Figure 2B). The results obtained through blood smears were highly correlated with those from flow cytometry (r = 0.61; $P < 0.001$; Figure

2C). Moreover we also found alterations in absolute white blood cell counts through flow cytometry. In particular, we found that exogenous CORT increased on average 1.09-fold the absolute granulocyte count ($F_{1,26} = 9.35$; $P < 0.005$; Figure 2D) but did not alter the absolute lymphocyte count ($F_{1,26} = 1.49$; $P = 0.271$; Figure 2D).

The intra-sample CV of lymphocyte proportion was on average 5.86 % for blood smears, and 12.71 % for flow cytometry. For the proportion of granulocytes, the intra-sample CV was on average 11.78 % for blood smears, and 14.72 % for flow cytometry. The inter-sample CV of proportion of lymphocytes was on average 10.82 % for blood smears and 14.32 % for flow cytometry. For the proportion of granulocytes, the inter-sample CV was on average 15.56 % for blood smears, and 18.71 % for flow cytometry. The intraclass correlation coefficient (ICC) indicated a higher consistency of the G:L ratio within treatments for flow cytometry (ICC = 0.74) than for blood smears (ICC = 0.66).

Discussion

Flow cytometry with non-specific lipophilic dyes as $DiOC_5(3)$ enabled detection of alterations in the immune state of amphibian larvae. Using both direct cell count in blood smears and flow cytometry we demonstrate a direct effect of CORT in producing an increased G:L ratio (Figure 2A and 2B, respectively), indicative of immunological suppression in larval amphibians. Our result for *Pelobates cultripes* tadpoles is in agreement with Falso et al. (2015) findings on *Xenopus laevis* and *Lithobates catesbeianus*, and is congruent with increased infection of larval treefrogs by trematodes after being treated with glucocorticoids (Belden and Kiesecker, 2005).

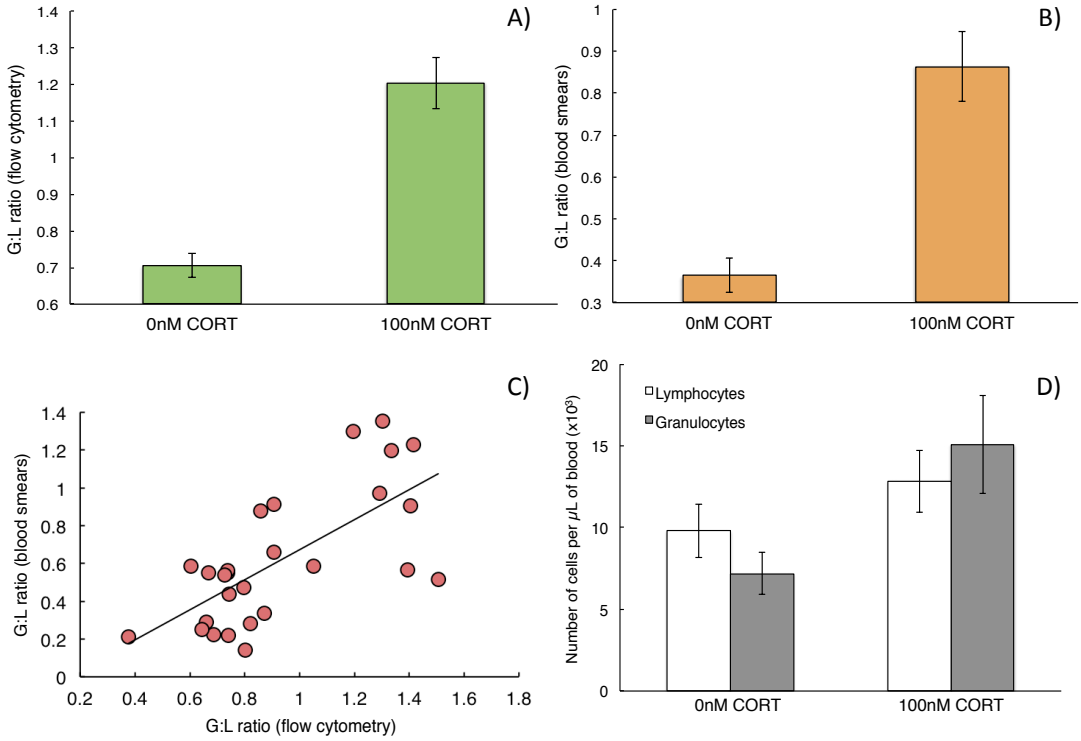

Figure 2. A) Granulocytes:Lymphocytes (G:L) ratio determined by flow cytometry in response to no exogenous (0 nM CORT) and to 100 nM corticosterone (100 nM CORT, stress induction) treatments. Bars indicate ± S.E. B) G:L ratio determined by blood smears in response to 0 and 100 nM corticosterone treatments. C) Correlation between the G:L ratio determined by flow cytometry or by direct cell count in blood smears (correlation coefficient = 0.61) D) Absolute count of lymphocytes (white) and granulocytes (grey) in response to 0 and 100 nM corticosterone treatments as determined by flow cytometry.

Both blood smears and flow cytometry are valid techniques for determining changes in G:L ratio, and the suitability of each one will depend on the experimental requirements, tractability, and the study system. A major difference between blood smears and flow cytometry lies in determining granulocyte types. Blood smears allow discrimination among granulocyte types (i.e. neutrophils, eosinophils, and basophils) whe-

reas flow cytometry does not because it uses non-specific dyes that lump all granulocyte types together (Uchiyama et al. 2005). This is not a big caveat for amphibian studies since granulocytes in amphibians are mostly represented by neutrophils (Davis et al. 2008) and therefore changes in G:L ratio largely reflect changes in the abundance of neutrophils. Flow cytometry has the advantage of providing absolute counts of white blood cells, allowing detection of lymphopenia or granulocytosis. Quantification of absolute leukocyte number with blood smears would require a huge time investment and can result inaccurate. The use of hematology analyzers, which yield a complete blood cell count, may also help rapidly quantify relative and absolute leukocyte counts. However, automatic counters commonly require ca. 30 µL of blood, which can limit their use in amphibian larvae. More importantly, these automated counters are calibrated to be species-specific and to date there is no commercial counter developed for amphibians. Flow cytometry has the advantage of the small amount of blood required (1 µL), whereas a single blood smear will require much more, close to 20 µL. Flow cytometry can record up to 102-103 leukocytes and 103-104 erythrocytes per µL of blood, increasing the statistical power of the analyses. This may help reducing the number of animals processed and allow determination of the immune state of a single individual over time through repeated blood extractions. As for the suitability of each technique in field studies, the use of flow cytometry for leukocyte determination seems to be limited by the need to use fresh blood to get a good cytometric determination. Blood to be used in blood smears quantification can be fixed with absolute methanol and later (i.e. some weeks later) can be stained in the laboratory. Therefore flow cytometry is a rapid, simple, and inexpensive technique that allows quan-

tification of absolute and relative leukocyte counts within a few minutes. However, blood smears seem preferable in field studies that require surveys in remote areas that prevent blood from being assayed within a few hours, or if different types of granulocytes are to be distinguished.

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DISCUSSION

Virtually all organisms can respond to environmental changes by modifying some aspects of their phenotype, whether behavior, morphology, physiology or development. Such plastic responses necessarily require the existence of mechanisms to detect environmental inputs and to develop the induced phenotype. The maintenance and activation of such mechanisms may incur in short and long term consequences for individuals, which could constrain the evolution of plastic responses. Particularly, amphibian species show a high degree of phenotypic plasticity in their behavior, coloration, physiology, morphology and life history, which probably has been favored by their low vagility and high philopatry (Scheiner, 2016; Edelaar et al. 2017). Most amphibian species have an early aquatic larval phase during which individuals metamorphose into terrestrial juveniles. The larval phase of amphibians involves profound anatomical changes and it is also a period in which individuals may feed and grow to store fat reserves and reach an adequate size to face their new way of life after metamorphosis. The size and weight, or body condition, of individuals after metamorphosis largely determine their survival odds in the short and mid-term, especially when juveniles face a long inactivity period of hibernation or aestivation after metamorphosis. In this thesis we investigated the physiological

mechanisms enabling developmental plasticity in amphibians during their larval phase, and its consequences for their postmetamorphic survival odds.

Production costs and consequences of plasticity

The neuroendocrine pathways underlying developmental plasticity in amphibian larvae have been well studied. They imply hypothalamic stimulations that produce hormones such as corticosterone and thyroid hormone, which lastly induce morphogenesis and developmental acceleration (Denver, 2009). This neuroendocrine activation normally occurs under stressful scenarios such as food deprivation or increased larval density (Crespi and Denver 2005; Belden et al. 2007), and involves metabolic alterations that can induce cellular oxidative stress and immunosuppression, although these consequences are not still fully well studied.

In this thesis I try to get a general understanding of the mechanisms involved in the responses of amphibian larvae to environmental changes. For this purpose, in Chapter 1 we studied alterations of corticosterone levels, metabolic rate, antioxidant activities, lipid peroxidation, and immune status in larvae exposed to different levels of an array of natural and anthropogenic environmental stressors: salinity, herbicide (glyphosate), water pH, predators (native and invasive), and temperature, which are considered potential stressors for larval amphibians. Our results denote the complexity of physiological stress responses since different stressors elicited a variety of hormonal, metabolic, or immunological responses that not always occurred in the same direction or to the same extent. High levels of salinity and glyphosate (i.e. 9ppt and 2 mg/L, respectively) caused the most profound physiological alterations in tadpoles, indicated

by increased corticosterone levels and metabolic rate, decreased glutathione reductase, or increased neutrophil-to-lymphocyte ratio. These alterations are congruent with the metabolic cascade activated by elevated levels of glucocorticoids (corticosterone is the main glucocorticoid in amphibians, birds and reptiles; Romero 2004). Glucocorticoids secretion enhances metabolism, which produces an excess of reactive oxygen substances (ROS) (Haussmann and Marchetto, 2010; Costantini et al. 2011) and diminish *de novo* synthesis of antioxidant enzymes (Kaushik and Kaur, 2003). The correlation between increased glucocorticoids production and oxidative stress was meta-analyzed by Costantini et al. (2011). This study showed that increased levels of glucocorticoids induced oxidative stress, although this effect depended on the treatment duration, being larger in long term experiments. Similarly, Davis et al. (2008), reviewing the relationship between glucocorticoids and immune status, found that stress hormones induced increases in the neutrophil-to-lymphocyte ratio, as well neutrophilia (decrease in the total neutrophils count) and lymphopenia (decrease in the total lymphocytes count). Neutrophils are the most abundant type of granulocytes in amphibians, being highly motile and having important anti-microbial functions (Amulic et al. 2012). However, there is controversy surrounding the interpretation of leukocyte parameters since the information obtained in blood smears does not explain by itself the ability of individuals to produce an immune response (Davis et al. 2008) and it is still unknown if immune alterations induced by elevated glucocorticoid levels indicate stress and illness or conversely show an active response to other causes, such as parasites. For instance, exogenous exposure to corticosterone did not alter susceptibility to a fungal pathogen (*Batrachochytrium dendrobatidis*) in three amphibian species (Searle et al. 2014), whereas *Plethodon shermani* salamanders treated with

corticosterone had higher infection by the same pathogen (Fonner et al. 2017). Further empirical studies should elucidate this paradox, and explain the causes for these species-specific differences.

On the other hand, we found decreased corticosterone levels in tadpoles exposed to native predators but not to invasive predators, which denotes a lack of hormonal response to invasive crayfish. This is congruent with previous behavioral and morphological observations in tadpoles showing that they are often not able to recognize invasive species as potential predators (Gomez-Mestre and Díaz-Paniagua, 2011; Polo-Cavia and Gomez-Mestre, 2014). Reduced corticosterone levels explain the reduction in activity rate that is commonly observed in tadpoles exposed to native predators, which is up to 57% (Polo-Cavia and Gomez-Mestre 2014). In the last years, some studies have also assessed the role of corticosterone on governing morphological responses observed in tadpoles exposed to predators, i.e. increases in tail depth and pigmentation (Hossie et al. 2010; Maher et al. 2013; Joshi et al. 2016). However, these morphological changes seem to be a by-product of the behavioral responses against predators, increasing tadpoles' survival rate (Chivers and Smith, 1998; Polo-Cavia and Gomez-Mestre, 2014). Our result also opens a debate around the definition of stress, since commonly elevated levels of glucocorticoids are associated with stressful events (Romero, 2004; Jessop et al. 2013). Corticosterone levels can be modulated in order to induce adaptive behavioral and morphological responses under stressful scenarios, as indicated decreased levels of this hormone in tadpoles exposed to predators, hence classical stress definition should be reconsidered.

In Chapter 1, corticosterone and the antioxidant enzyme glutathione reductase were the most sensitive parameters to stress. Beyond the importance of knowing physiological alterations of plasticity, these

results might provide relevant insights for conservation, ecological, and evolutionary studies.

Cascading physiological responses to changing environments, as those studied in Chapter 1**,** can inhibit growth or fecundity of individuals, affecting their life-history traits and ultimately their survival (Haussmann and Marchetto, 2010; Crespi et al. 2013). In Chapter 2 we observed that pond drying and predator presence during larval development alter the short and long term odds of survival of spadefoot toad tadpoles. Both factors decreased tadpole survival and altered larval development and growth in different ways and to different extent, resulting in variation in the amount of accumulated fat reserves, oxidative stress, or telomere length of metamorphs. Pond drying reduced metamorph body size and also their fat reserves. Body size and fat storage of juvenile amphibians are good predictors of their survival odds to first reproduction (Pechenik, 2006; Scott et al. 2007). In our study area, juvenile spadefoot toads enter into aestivation immediately after metamorphosis. Thus body condition at the end of metamorphosis is a reliable indicator of short-term survival, although it might be reversible under optimal environmental conditions after metamorphosis such as high food availability and moderate temperatures. On the other hand, predators reduced intraspecific competition for resources in tadpoles. This allowed surviving individuals to grow more and to accumulate large fat reserves, which is expected to increase short-term survival odds of juveniles. However, enhanced growth required an intense cellular metabolism and induced oxidative stress, and also involved telomere shortening in juvenile amphibians. Enhanced growth also would reduce long term expectancies of toad survival since both reactive oxygen substance (ROS) production and telomere shortening correlates with decreased lifespan (Monaghan and Haussmann 2006; Dowling and Simmons

2009). Our results link with the theory of caloric restriction (McCay et al. 1935; Sinclair, 2005). This theory suggests that restricting food intake without incurring in malnutrition extends life by slowing ageing processes, although mechanisms responsible for life extension are still not well known (Masoro, 2005). Events occurring early during development can have important consequences for later stages, particularly in species with complex life-cycles (Pechenik, 2006). For instance, previous studies described that organisms experiencing oxidative stress and telomere shortening during early development dramatically experience cellular senescence and reduce lifespan (Hall et al. 2004; Metcalfe and Alonso-Alvarez, 2010; Heidinger et al. 2012). The results showed in **Chapter 2** indicate that common risks for amphibian larvae, such as pond drying and predator presence, alter intraspecific interactions and involve drastic alterations on life-history traits that might play a key role in determining short-term survival and fitness of individuals. However, implications for lifespan of oxidative stress and telomere shortening experienced by tadpoles should be confirmed through long term studies.

Maintenance costs of plasticity

Evolutionary models argue that plasticity might be reduced by costs of maintaining the machinery needed to detect, monitor, and respond to environmental inputs (DeWitt 1998; Auld et al. 2010). However, costs of plasticity often seem to be mild or, at least, hard to detect (Van Buskirk and Steiner 2009; Murren et al. 2015). A possible explanation to the apparent lack of such costs is that selection might act by purging them, as for example removing interactions between loci involved in plasticity and loci affecting fitness (Murren et al. 2015), although costs can re-emerge after recombination between genotypes

(Van Kleunen and Fischer, 2007). Probably, broad fitness-related phenotypic consequences of plasticity are buffered over evolutionary time but subtle costs persist, hence molecular approaches might help to detect maintenance costs of plasticity. For this purpose, in Chapter 3 we tested for the existence of physiological maintenance costs of plasticity in amphibian larvae in response to pond drying and predators by quantifying metabolism and immune parameters. We found that genotypes capable of varying their development, growth and morphology to a greater extent in response to environmental induction (i.e. more plastic genotypes) showed constitutively higher activities of the enzyme glutathione peroxidase (GPx). Antioxidant roles of GPx result essential for protecting cell from oxidative damage, since GPx detoxifies H_2O_2 molecules that are overproduced during enhanced mitochondrial respiration (Murphy, 2009). Similarly, increased activity of the enzyme superoxide dismutase and also elevated lipid peroxidation appeared to be costly mechanisms in terms of plasticity maintenance. Therefore, we detected signs of cellular metabolic costs associated with highly plastic genotypes. Antioxidant enzymes seem to regulate cellular ageing, indicated by empirical studies in which induced overexpression of antioxidant enzymes extend the life of organisms (Landis and Tower, 2005; Schriner et al. 2005), although these mechanisms are still not fully understood (Lu and Finkel, 2008; Liochev, 2013). Also, genotypes with high plasticity in growth when exposed to predator cues showed high granulocyte-to-lymphocyte ratio. As indicated above, elevated values of this ratio seem to be linked to high disease susceptibility (Huff et al. 2005) and to poor body condition or health of individuals (Gomez et al. 2008; Lobato et al. 2009). The detection of maintenance costs of plasticity through physiological measurements matches predictions made by Auld et al. (2010), indicating that probably organisms invest resources

in maintaining the physiological machinery needed to have the ability to be plastic. Such maintenance costs were previously estimated through indirect measurements of fitness such as body size, growth, survival, or time to maturity (Scheiner and Berrigan, 1998; Relyea, 2002). In our opinion, results from Chapter 3 encourage physiological approaches to the study of plasticity costs because it allows detection of subtle but important metabolic costs.

Also in Chapter 3 we found an intriguing trade-off between the ability of larvae to accelerate or delay their development in response to pond drying and predators, respectively. The ability to plastically induce rapid morphogenesis and faster development results adaptive when ponds begin to dry out (Denver et al. 1998). On the other hand, larvae exposed to predators normally slow-down their development, which is a consequence of the adaptive reduction of their activity rate (Laurila et al. 2004). In amphibian larvae, developmental plasticity is regulated by glucocorticoids and thyroid hormone whose production regulates processes such as morphogenesis, developmental timing or metabolic rate (Denver, 2009; Hossie et al. 2010; Gomez-Mestre et al. 2013). Hence, responses to both pond drying and predators seem to be mediated by the same pathways, but they act in different directions, which might limit the evolution of such responses.

Mechanisms underlying adaptive plasticity evolution

In a changing world, organisms are continuously responding to diverse external inputs in order to increase their survival odds. For instance, several adaptations to novel urban environments have been described in many bird species, which commonly explore new habitats to occupy free-niches (Gil and Brumm, 2013). These adaptations involve physiological alterations such as adjustments in

corticosterone levels (Atwell et al. 2012) that regulate activity rate or vocalizations that allow a better match with the local environment (Breuner et al. 1998). In Chapter 4 we study adaptive physiological divergence in populations of frogs inhabiting islands that have different types of breeding ponds regarding water permanence: islands with only temporary ponds, islands with only permanent ponds, and islands with both types of ponds. We found that populations from islands with only temporary ponds had a higher degree of developmental plasticity, and that these populations showed lower constitutive antioxidant activity of enzymes such as catalase and glutathione reductase. They also showed shorter telomeres. Differences in antioxidant responses among populations would indicate metabolic costs associated with high developmental plasticity. Lower activities of these enzymes might indicate an enzymatic inactivation caused by an excess of ROS produced during developmental acceleration (D'Autréaux and Toledano, 2007; Monaghan et al. 2009). However, an alternative explanation might be that selection favored individuals that maximized mitochondrial respiration (Salin et al. 2015), hence producing less ROS when they experienced higher metabolic rates and showed lower antioxidant activity (Salin et al. 2015). Altered antioxidant responses together with shortened telomeres might compromise health and lifespan of individuals, which could have cascading effects on populations, altering their demography and viability. Shortened telomeres observed in more plastic populations might be also a consequence of an early reproduction since differences in telomere length among populations have been associated to trans-generational effects of male age at reproduction (Kimura et al. 2008; Eisenberg et al. 2012). Moreover, telomere length during initial ontogenetic stages is very important because it correlates well with overall lifespan (Heidinger et al. 2012).

Synthesis and future challenges

In this thesis we found that plastic responses to changing environments in amphibian larvae require profound physiological adjustments, which might have important short-term and long-term consequences. We also detected physiological costs associated to more plastic genotypes that can explain within-species differences in plasticity. Along this line, we found trade-offs between opposed developmental responses regulated by the same neuroendocrine pathways, posing constraints on the evolution of both responses. Finally, we demonstrated that populations expressing different degrees of developmental plasticity experienced unequal levels of metabolism-related physiology, which we understand as costs paid by organisms to maintain high levels of plasticity.

This thesis increases the available information about physiological mechanisms underlying causes and consequences of plasticity, and highlights the importance of physiological approaches to the understanding of the evolution of plasticity. The use of genomic and physiological tools should help to understand speciation and diversification processes, since plastic alterations in gene expression and ultimately in physiology of organisms are necessary to deal with new or changing environments. On the other hand, consequences of plasticity for lifespan and fitness of individuals should be addressed via a combination of field and laboratory experiments, although for this purpose amphibians do not constitute an ideal group due to their longevity and the long time they require to reach sexual maturity. Finally, the inheritance of physiological mechanisms underlying plasticity should be inspected in future studies by using species with high generation rate or by investigating populations inhabiting natural systems in which a limited number of factors were affecting the evolution of plasticity.

Conclusions

1 Non-lethal levels of potential stressors, such as salinity, herbicide, water pH, native and non-native predators, and temperature induced changes in corticosterone level, metabolic rate, activity of various antioxidant enzymes, lipid peroxidation, or immune state of spadefoot toad larvae (*Pelobates cultripes*).

2 High levels of salinity and herbicide caused dramatic physiological alterations in terms of increased corticosterone levels, metabolic rate, antioxidant activity, or immunosuppression in tadpoles. Moreover, tadpoles recognized the presence of native predators and reduced their corticosterone levels accordingly, whereas they did not recognize non-native predatory crayfish, *Procambarus clarkii*.

3 Corticosterone and the antioxidant enzyme glutathione reductase were the most sensitive parameters against the studied factors. Therefore both parameters might be good candidates for use in physiological monitoring of natural populations.

4 Pond drying and predators induced changes in life-history traits of *P. cultripes* larvae and involved noticeable physiological alterations. Pond drying accelerated tadpole development, but at the expense of metamorphosing smaller and reducing fat reserves. However, cellular oxidative stress was successfully buffered by increased antioxidant activity, and telomeric regions remained unaltered. On the other hand, predators greatly reduced larval density, allowing survivors to grow fast, reach a bigger size at metamorphosis, and accumulate large fat reserves. These fast-growing individuals, however, showed signs of oxidative stress and had shorter telomeres.

5 Pond drying and predators may involve short and long-term consequences for juvenile amphibians. Responses to pond drying reduce the odds of short-term survival because result in depletion of fat reserves, although this may be reversible in the long run. Individuals surviving predators grew at a high rate shortening their telomeres, and likely reducing their lifespan.

6 Plasticity against pond drying and predators is limited by the costs of physiological maintenance in *P. cultripes* tadpoles. Variations in the degree of developmental, growth, and morphological plasticity among genotypes (families) are linked to high constitutive values of antioxidant enzyme activities, lipid peroxidation, granulocyte-to-lymphocyte ratio, or growth rate. Such physiological costs would limit the evolution of reaction norms.

7 Developmental adaptive plasticity can also be limited by trade-offs due to simultaneous exposure to factors inducing opposite responses. Those trade-offs were detected in terms of larval developmental plasticity, because genotypes (families) that accelerated their development in response to pond drying could not delay the time to metamorphosis when responding to predators.

8 Environmental heterogeneity can affect the degree of adaptive plasticity in amphibian larvae. We found that populations of *Rana temporaria* in the Swedish islands system, exposed during each breeding season to high risk of pool desiccation, showed a higher degree of developmental plasticity compared to populations inhabiting islands with low or variable risk of desiccation. Those

highly plastic populations experienced physiological alterations of increased plasticity, such as reduced activity of two antioxidant enzymes (catalase and glutathione reductase) and shortened telomeres.

9 Low enzymatic activities observed in *Rana temporaria* populations showing high developmental might indicate exhaustion of these enzymes but also a maximized mitochondrial respiration. However, net reduction in the telomeric regions would indicate a metabolic toll paid by these populations, which might have important consequences for long-term survival of organisms.

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Esta tesis tampoco hubiera sido posible si no existiera vida inteligente extra muros. Gracias a mis queridos Yonkis Tarantinos: inmortales y robustos como los buenos robles. ¿Qué sería de nosotros sin las reglas Thinator o sin los debates de Juanra&Manolito sobre jugadas de poker? Gracias Fairy Verde, en otro orden de cosas, por ser mi chófer con o sin carnet, en España o en la Costa Oeste. Gracias, por tantas otras cosas también. (…). Gracias a Gelu y a Draker, y por qué no, gracias también a Guille. Gracias a Simón, Luis, Villa y Ale. Gracias a mis compañeros de pádel (Luis, Martín, Juan Antonio, José …), que consiguen que durante una hora y media a la semana pueda desfogar y, si hay suerte, irme a casa con una victoria. Aquí es donde me paro a agradecer a Cristina Ramos por haberme animado a jugar, a volver a darle a la raqueta, que en realidad se llama pala. Gracias a ella y a Benjamín por ser buenas personas, quedan pocas así. Gracias a mis compañeros musicales, a Pakoh, Flak, Felipe. Ellos me devolvieron a la música, tocar con vosotros cada tarde-noche de jueves y grabar todas esas jam-sessions han sido de los mejores recuerdos que me voy a llevar para no parar de escuchar cuando esté lejos. Hay muchas cosas que el dinero no puede comprar. Gracias a mis

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GRACIAS.

Hace unos meses tenía pensado que esta fuera una tesis con anti-agradecimientos, esos "dedicados" a todas aquellas malas personas que degradan este mundo y lo poquito que nos queda del medio ambiente. Para los que hacen que a veces sea imposible vivir de la ciencia aunque te dediques en cuerpo y alma a esto, aunque quieras aportar algo nuevo en cualquiera área del conocimiento trabajando a destajo. Sin embargo, al poco me di cuenta de que eso sería ensuciar estas pocas páginas cargadas de gloria momentánea, la ocasión de disfrutar con todo lo bueno que nos rodea, que por suerte es mucho. Es por ello por lo que sólo quería aprovechar para agradecer una vez más a todas y cada una de las personas que han pasado por este largo camino en la EBD (y fuera de ella) que acaba aquí o que se postpone hasta nuevo aviso. Cuando llegué jamás imaginé un balance tan positivo. Se que en buena parte hay un factor "suerte" que hace que esto sea así cuando una persona llega a un nuevo sitio a ciegas. Sin embargo, y como no termino de creer en en esa suerte, todo fue por culpa vuestra. "Nos volveremos a ver, porque siempre hay regreso."